8th International Congress of Veterinary Virology

EUROPEAN SOCIETY FOR VETERINARY VIROLOGY

20 years of ESVV: Integrating classical and molecular virology

Programme & Proceedings

Budapest – Hungary

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8th International Congress of Veterinary Virology
Budapest, Hungary, 23–26 August 2009

20 years of ESVV: Integrating classical and molecular virology

Programme and Proceedings

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Proceedings edited by
Mária Benkő and Balázs Harrach

Editing help and book cover design by
Győző Kaján, Márton Vidovszky, Endre Kovács, Máté Jánoska, Laura Pantó, Andor Doszpoly

Veterinary Medical Research Institute, Hungarian Academy of Sciences
H-1143 Budapest, Hungária krt. 21., Hungary
e-mail: esvv2009@vmri.hu, harrach@vmri.hu
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Preface

Dear Colleagues,

Welcome to the 8th International Congress of the ESVV in Budapest. The subtitle of this anniversary congress is "*20 years of ESVV: Integrating classical and molecular virology*". The lectures and the posters will summarize the two-decade history of the Society and will illustrate the recent achievements in veterinary virology.

The science of veterinary virology has a long and very interesting history. For example, in 1897 and 1898 the German scientists Friedrich Loeffler and Paul Frosch were the first to describe the filterability of an animal virus. This pioneering work on the foot-and-mouth disease virus and on the interesting scenario of this highly infectious and devastating viral disease of various animal species led to the establishment of a range of famous high security veterinary research institutes in Europe, such as the laboratories of Insel Riems, Pirbright and Lindholm. Simultaneously, veterinarians in other countries of Europe have started to study a range of other infectious maladies at the end of the 19th century and in the early years of the 20th century and described a range of viral diseases in various host species.

The host institution of our 8th International Congress, the Faculty of Veterinary Science in Budapest, which is one of the oldest veterinary schools in Europe, founded in 1787, has also serious input in the history of veterinary virology. For example, Aujeszky's disease was described here by professor Aladár Aujeszky in 1902, and Marek's disease by professor József Marek in 1907. Subsequently, a range of important investigations was reported from this veterinary school and famous textbooks on infectious diseases, written by Professors Ferenc Hutÿra, József Marek and Rezső Manninger, were translated to dozens of languages and used in veterinary education worldwide. It is important to mention the research activities of professor Adorján Bartha, a previous, very active member of our Society, who had a worldwide-recognised input into veterinary virology, and studied the infection biology of adenoviruses, herpesviruses, and the a range of other viruses in different host species, by establishing a "school of veterinary virologists".

Let's hope that the 8th International Congress of Veterinary Virology will further strengthen the input of the ESVV in various aspects of veterinary virology. The congress will cover a wide range of "hot" subjects and it will provide plenty of facilities for the participants for interesting scientific discussions in the quiet historical atmosphere of the old veterinary school.

On behalf of the organising and scientific committees I would like to thank all participants for their contribution to the Congress. I hope you will enjoy the meeting and I wish you a pleasant stay in Budapest.

Sándor Belák
President of the ESVV

Programme at a glance

Sunday August 23

14:00-20:00: Registration, Display of posters	
17:00-18:40	18:45-22:00
Opening session	Welcome reception

Monday August 24

8:15-10:15	10:15-10:45	10:45-13:00	13:00-14:00	14:00-16:00	16:00-17:00	17:00-22:00
Plenary Session	Coffee break	***Emerging viruses & zoonoses I.*** (Main Hall) ***Vaccines & viral immunology*** (Lecture Hall)	Lunch	***Host-virus interactions & pathogenesis*** (Main Hall) ***Epizootiology*** (Lecture Hall)	***Poster viewing***	Excursion to the Lázár Equestrian Park (horse show and traditional Hungarian dinner)

Tuesday August 25

8:15-10:15	10:15-10:45	10:45-13:00	13:00-14:00	14:00-16:00	16:00-17:00	17:00-18:00	19:30-22:30
Plenary Session	Coffee break	***Viral diagnosis*** (Main Hall) ***Viruses of wild and exotic animals*** (Lecture Hall)	Lunch	***Emerging viruses & zoonoses II.*** (Main Hall) ***New developments*** (Lecture Hall)	***Poster viewing*** Coffee break	***General assembly of the ESVV***	Gala Reception at the Hungarian Agricultural Museum (Vajdahunyad Castle)

Wednesday August 26

8:30-11:10	11:10-11:40	11:40-13:10	13:15-13:25	13:30-15:30	15:30-17:00
Plenary Session	Coffee break	***Evolution & genomics*** (Main Hall) ***Miscellaneous topics*** (Lecture Hall)	Closing remarks	Farewell lunch	Removal of posters

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Programme

August 23 (Sunday)

14:00-20:00 **Registration** (Mounting of Posters)

17:00-18:40 Opening Session (Chairs: **Mária Benkő** & **André Jestin**)

17:00-17:20 Opening Words by **Sándor Belák** (President of ESVV) and **László Fodor** (Dean of the Faculty of Veterinary Science, Budapest)

17:20-18:00 Veterinary virology in the real world (the Zvonimir Dinter Memorial Lecture; 2009)
Steven Edwards

18:00-18:40 Biodiversity of mammals, birds, and viruses
Paul-Pierre Pastoret

18:45-22:00 *Welcome reception*
Sponsored by

August 24 (Monday)

8:15-10:15 Plenary Session (Chairs: **Marian C. Horzinek** & **Éva Nagy**)

8:15-8:55 A live attenuated vaccine against feline infectious peritonitis (FIP) protecting by cellular rather than virus-neutralizing immunity
Bert Jan Haijema, Pepijn Schellen, Herman Egberink, Peter J. M. Rottier

8:55-9:35 An educated guess on the pathogenic forces behind sheep-associated malignant catarrhal fever
Mathias Ackermann

9:35-10:15 Mechanisms of bluetongue virus evolution and their potential relevance to virus phenotypic properties and diagnostic and vaccine strategies
N. James Maclachlan

10:15-10:45 *Coffee break*

10:45-13:00 Parallel Sessions:
Main Hall: **Emerging viruses & zoonoses I.**
Lecture Hall: **Vaccines & viral immunology**

10:45-13:00 Emerging viruses & zoonoses I. (Main Hall)
(Chairs**: Marc Eloit** & **Carlos Martins**)

10:45-11: 00 Toggenburg orbivirus: genetic characterization, diagnostics and epidemiology of a novel bluetongue virus detected in goats from Switzerland
Martin Hofmann, Valérie Chaignat, Gabriella Worwa, Barbara Thuer

11:00-11:15 Bluetongue virus in Northern and Western Europe: Vaccination strategies and protection in sheep
Chris Oura, Toby Floyd, James Wood, Carrie Batten

11:15-11:30 Detection of bluetongue serotype 1 in red deer (*Cervus elaphus*) by RT-PCR
Belén Rodríguez-Sánchez, Christian Gortázar, Francisco Ruiz-Fons, José M. Sánchez-Vizcaíno

11:30-11:45 Experimental infection of red deer (*Cervus elaphus*) with bluetongue virus serotypes 1 and 8
Jorge Ramón López-Olvera, Caterina Falconi, Paloma Férnandez-Pacheco, Jovita Fernández-Pinero, Miguel Ángel Sánchez, Agustín Palma, Irene Herruzo, Joaquín Vicente, Miguel Ángel Jiménez-Clavero, Marisa Arias, José Manuel Sánchez-Vizcaíno, Christian Gortázar

11:45-12:00 Infection dynamics of the current novel 'swine-like' human influenza A/H1N1 in pigs: A/California/07/09
Sharon Brookes, Alejandro Nunez, Derek Clifford, Steve Essen, Richard Irvine, Kristien Van Reeth, Gaelle Kuntz-Simon, Willie Loeffen, Emanuela Foni, Lars Larsen, Mikhail Matrosovich, Michel Bublot, Jaime Garcia, Martin Beer, Giovanni Cattoli, Ian Brown

12:00-12:15 Contact exposure experiments using the new influenza A/H1N1 virus
Elke Lange, Donata Kalthoff, Ulrike Blohm, Timm Harder, Jens Teifke, Angele Breithaupt, Christina Maresch, Thomas Mettenleiter, Martin Beer, Thomas Vahlenkamp

12:15-12:30 Geographic spread of an encephalitic lineage 2 West Nile virus strain in 2008 in Central Europe
Tamás Bakonyi, Károly Erdélyi, Emőke Ferenczi, Orsolya Kutasi, Bernhard Seidel, Herbert Weissenböck, Norbert Nowotny

12:30-12:45 Characterization of the equine West Nile virus outbreak in Hungary in 2008
Sylvie Lecollinet, Orsolya Kutasi, Sara Sardi, Imre Biksi, Céline Bahuon, Tamás Bakonyi, Stéphan Zientara

12:45-13:00 Newcastle disease virus in West Africa: new virulent strains identified in non-commercial farms
Chantal J. Snoeck, Mariette F. Ducatez, Ademola A. Owoade, Olufemi O. Faleke, Bello R. Alkali, Marc C. Tahita, Zekiba Tarnagda, Jean-Bosco Ouedraogo, Issoufou Maikano, Patrick Okwen Mbah, Jacques R. Kremer, Claude P. Muller

10:45-13:00 Vaccines & viral immunology (Lecture Hall)
(Chairs: **Thierry van den Berg** & **Vilmos Palya**)

10:45-11:00 Recombinant Newcastle disease viruses as marker vaccines against highly pathogenic avian influenza
Angela Römer-Oberdörfer, Eylin Topfstedt, Christian Grund, Daniela Deckers, Miriam Rudolf, Jutta Veits, Thomas C. Mettenleiter

11:00-11:15 Development of fowl adenovirus 9 based vector vaccine expressing the hemagglutinin gene of an H5N1 influenza virus
Dan-Hui Yang, Juan C. Corredor, Peter J. Krell, Éva Nagy

11:15-11:30 Immunogenicity of fowlpox vectored and inactivated avian influenza vaccines in chickens with maternal antibodies
Alexandra Richard-Mazet, Mieke Steensels, Sandrine Chanavat-Bizzini, François-Xavier Le Gros, Michelle Duboeuf, Bénédicte Lambrecht, Thierry van den Berg, Michel Bublot

11:30-11:45 Efficacy of C-strain vaccine in domestic pigs challenged with genotype 2.1 and 3.3 classical swine fever virus shortly after vaccination
Helen E. Everett, Simon P. Graham, Derek Clifford, Helen Johns, Felicity J Haines, Olubukola Sosan, F. Javier Salguero, Trevor W. Drew, Helen R. Crooke

11:45-12:00 Evaluation of a new vaccination strategy against bluetongue based on expressing a group specific antigen (VP7)
Coraline Bouet-Cararo, Vanessa Contreras, Annie Fournier, Marion Szelechowski, Emmanuel Breard, Pierre Russo, Richard Thiery, Isabelle Schwartz-Cornil, Stéphan Zientara, Bernard Klonjkowski

12:00-12:15 Evaluation of the response induced by VLP vaccines for bluetongue virus serotypes 1 and 1&4 in sheep
Ana C. Pérez de Diego, Belén Rodríguez, Rob Noad, T. N. Athmaran, Meredith Stewart, José M. Sánchez-Vizcaíno, Polly Roy

12:15-12:30 Determination of the minimum protective dose for bluetongue serotype 2, 4 and 8 vaccines in sheep
Jacob Modumo, Estelle H. Venter

12:30-12:45 Evaluation of vaccines against bluetongue virus in a murine model
Javier Ortego, Noemí Sevilla, Teresa Rodríguez-Calvo, Juan Anguita, Eva Calvo-Pinilla

12:45-13:00 Heterologous prime boost vaccination with DNA and recombinant modified vaccinia virus Ankara expressing VP2, VP5, and VP7 proteins of bluetongue virus protects IFNAR(-/-) mice against bluetongue infection
Eva Calvo-Pinilla, Teresa Rodríguez-Calvo, Noemí Sevilla, Javier Ortego

13:00-14:00 Lunch

14:00-16:00 Parallel Sessions
Main Hall: **Host–virus interactions & pathogenesis**
Lecture Hall: **Epizootiology**

14:00-15:45 Host–virus interactions & pathogenesis (Main Hall)
(Chairs: **Thomas Mettenleiter** & **Etienne Thiry**)

14:00-14:15 Pre-infection of pigs with *Mycoplasma hyopneumoniae* enhances the pathogenesis of H1N1 but not H1N2 European swine influenza virus
Gaëlle Kuntz-Simon, Stéphane Gorin, Stéphane Quéguiner, Céline Deblanc, Anne Gautier-Bouchardon, Séverine Ferré, Nadia Amenna, Gérard Bénévent, Pierre Ecobichon, Jean-Marie Guionnet, Nathalie Thomas, Roland Cariolet

14:15-14:30 Pathogenicity of selected fowl adenovirus isolates
Helena Grgić, Dan-Hui Yang, Sheila Watson, Éva Nagy

14:30-14:45 Species-specific contribution of the four C-terminal amino acids of influenza A NS1 protein to virulence
Sébastien M. Soubies, Christelle Volmer, Guillaume Croville, Pierrette Coste, Caroline Lacroux, Josianne Loupias, Brigitte Peralta, Jean-Luc Guérin, Romain Volmer

14:45-15:00 Avian influenza viruses use different receptors for infection of the respiratory epithelium in different avian host species
Maren Bohm, Christine Winter, Georg Herrler

15:00-15:15 Assembly and maturation of pestiviruses
Stefanie Schmeiser, Matthias König, Heinz-Jürgen Thiel

15:15-15:30 Experimental infection of calves with BTV-8 using two different inocula
Ludovic Martinelle, Fabiana Dal Pozzo, Pierre Sarradin, Ilse De Leeuw, Kris De Clercq, Dominique Ziant, Etienne Thiry, Claude Saegerman

15:30-15:45 Bovine immunodeficiency virus infection fails to provide protection against subsequent Jembrana disease virus infection
Tegan J. McNab, Moira Desport, I. W. Masa Tenaya, Nining Hartaningsih, Graham E. Wilcox

14:00-16:00 Epizootiology (Lecture Hall)
(Chairs: **Wim H. M. van der Poel** & **Miklós Rusvai**)

14:00-14:15 Potential role of ticks as vectors of bluetongue virus
Chantal Bouwknegt, Piet A. van Rijn, Jacqueline J. M. Schipper, Dennis Hölzel, Jan Boonstra, Ard M. Nijhof, Eugène M. A. van Rooij, Frans Jongejan

14:15-14:30 Determination of the role of hard (ixodid) ticks in the transmission of lumpy skin disease virus in cattle
Eeva S.M. Tuppurainen, Hein W. Stoltsz, Milana Troskie, David B. Wallace, Chris Oura, Philip S. Mellor, Koos (Jaw) Coetzer, Estelle H. Venter

14:30-14:45 Seroprevalence of equine viral arteritis in Croatia – variable specificity of imunnoenzime assay in different horse populations
Ljubo Barbic, Nenad Turk, Zoran Milas, Vilim Staresina, Zrinka Stritof, Josipa Habus, Vladimir Stevanovic, Josip Madic

14:45-15:00 Identification of canine coronavirus type I in a naturally infected cat
Sophie Le Poder, Lidia Duarte, Annie Fournier, Cristina Rebega, Carine Pinhas, Marc Eloit

15:00-15:15 An individual-based model for porcine circovirus type 2 (PCV-2) within-herd dynamics of infection
Mathieu Andraud, Béatrice Grasland, François Madec, André Jestin, Nicolas Rose

15:15-15:30 Influence of suckling pig convalescence from infectious diarrhea on their susceptibility to respiratory pathogens during growing period
Sergey A. Kukushkin, Taufik Z. Baybikov

15:30-15:45 Detection of six honeybee viruses in colonies affected with colony collapse disorder
Ivan Toplak, Danijela Rihtarič, Peter Hostnik, Jože Grom, Magdalena Vidmar

15:45-16:00 Honeybee viruses in Spain: diagnosis and distribution. Are they a problem?
Deborah Kukielka, José Manuel Sánchez-Vizcaíno

16:00-17.00 **Poster Session I:** Presenters of the posters in the topics listed below, are asked to be so kind as to stay at their poster: **Emerging viruses & zoonoses; Epizootiology; Evolution & genomics; Host-Virus interactions & pathogenesis; Viruses of wild & exotic animals.**

16:30-17:00 Coffee break

17:00-22:00 *Excursion to the* **Lázár Equestrian Park** (Horse show and traditional Hungarian dinner)
The horse show is sponsored by

Applied Biosystems

August 25 (Tuesday)

8:15-10:15 **Plenary Session** (Chairs: **Volker Moennig** & **N. James Maclachlan**)

8:15-8:55 Recent trends in the diagnosis of emerging and re-emerging transboundary animal diseases and in the detection of food- and waterborne infections
Sándor Belák

8:55-9:35 Circoviruses - a comparative approach
Daniel Todd

9:35-10:15 African swine fever: A dangerous remerging disease
José-Manuel Sánchez-Vizcaíno

10:15-10:45 *Coffee break*
10:45-13:00 **Parallel Sessions:**
Main Hall: **Viral diagnosis**
Lecture Hall: **Viruses of wild and exotic animals**

10:45-13:00 **Viral diagnosis** (Main Hall)
(Chairs: **Sándor Belák** & **Stefan Vilček**)

10:45-11:00 Microarray potential in diagnosis of infectious diseases
Akbar Dastjerdi, Rajesh Gurrala, Nick Johnson, Sylvia Grierson, Francesca Martelli, Nadia Inglese, Falko Steinbach, Malcolm Banks

11:00-11:15 New real-time PCR using Universal Probe Library (UPL) for detection of African swine fever virus (ASFV)
Jovita Fernández-Pinero, Carmina Gallardo, Ana Robles, Covadonga Pérez, Concepción Gómez, Raquel Nieto, Marisa Arias

11:15-11:30 A sensitive one-step real-time PCR technology used in veterinary virology and disease control
Szabolcs Kökény

11:30-11:45 Recombinant NS3 and monoclonal antibodies for serodiagnosis of pestiviruses infection by competitive ELISA
Giulia Pezzoni, Lidia Stercoli, Paolo Cordioli, Emiliana Brocchi

11:45-12:00 RT-PCR detection and phylogenetic analysis of Hungarian equine infectious anaemia virus strains
Miklós Rusvai, Tamás Bakonyi, Ákos Hornyák, Gyula Balka, Aymeric Hans, Norbert Nowotny

12:00-12:15 Validation of novel real-time PCRs for the simultaneous diagnosis of three different viral encephalomyelitis infections of horses
Marcello Sala, Antonella Cersini, Armando Damiani, Maria Teresa Scicluna, Giuseppe Manna, Valentina Spallucci, Andrea Caprioli, Maria Ilaria Ciabatti, Gian Luca Autorino

12:15-12:30 *In situ* proximity ligation assay for studying virus and pathogenesis
Jonas J. Wensman, Karl-Johan Leuchowius, Anna-Lena Berg, Liv Bode, Hanns Ludwig, Sándor Belák, Ulf Landegren, Ola Söderberg, Mikael Berg

12:30-12:45 Development of microsphere-based immunoassay for detection of antibodies to bovine viral diarrhea virus
Hongyan Xia, Lihong Liu, István Kiss, Ann Nordengrahn, Ronnie Eriksson, Jonas Blomberg, Malik Merza, Sándor Belák

12:45-13:00 Development of an RT-PCR test for chicken astroviruses
Victoria J. Smyth, Heather L. Jewhurst, Brian M. Adair, Daniel Todd

10:45-13:00 Viruses of wild and exotic animals (Lecture Hall)
(Chairs: **Silvia Blahak** & **Balázs Harrach**)

10:45-11:00 Detection and partial characterization of paramyxoviruses from reptiles
Rachel E. Marschang, Maha D. A. Shukur, Michael Pees, Volker Schmidt, Jürgen Seybold, Jens W. Frost, Silvia Blahak, Tibor Papp

11:00-11:15 New squamatid adenoviruses: partial PCR-sequence characterisation of the first lizard isolates together with other snake and lizard field samples
Tibor Papp, Beth Fledelius, Volker Schmidt, Győző L. Kaján, Inna Romanova, Rachel E. Marschang

11:15-11:30 The phylogenetic analysis of avipoxviruses in New Zealand birds
Hye-jeong Ha, Laryssa Howe, Maurice Alley, Brett Gartrell

11:30-11:45 Association of avian bornavirus with tissue lesions in psittacines with proventricular dilatation syndrome
Herbert Weissenböck, Karin Sekulin, Tamás Bakonyi, Felix Ehrensperger, Norbert Nowotny

11:45-12:00 Identification of a novel herpesvirus in bat using improved RDV method
Shumpei Watanabe, Ken Maeda, Kazuo Suzuki, Kentaro Kato, Yukinobu Tohya, Tetsuya Mizutani, Hiroomi Akashi

12:00-12:15 Detection, diversity and epidemiology of marine mammal astroviruses
James F.X. Wellehan, Rebecca Rivera, Stephanie Venn-Watson, Cynthia Smith, Eric Jensen, Frances M.D. Gulland, Hendrik Nollens

12:15-12:30 Novel marine mammal viruses: implications for human and animal virus ecology
Hendrik H. Nollens, Rebecca Rivera, Stephanie Venn-Watson, James F. X. Wellehan

12:30-12:45 Molecular characterization of a novel coronavirus, inducing feline infectious peritonitis (FIP)-like lesions in ferrets
Annabel G. Wise, Matti Kiupel, April K. Clark, Roger K. Maes

12:45-13:00 Shrews carry a variety of novel hantaviruses: evidence for unusual biological characteristics, compared to rodent-borne hantaviruses
Norbert Nowotny, Ralf Dürrwald, Jolanta Kolodziejek, Tamás Bakonyi, Armin Deutz, Herbert Weissenböck, Hans Homola, Helga Lussy, Tibor Csörgő, Gábor Rácz, Emőke Ferenczi, Barbara Herzig, Boris Klempa, Detlev H. Krüger

13:00-14:00 Lunch

14:00-16:00 Parallel Sessions
Main Hall: **Emerging viruses & zoonoses II.**
Lecture Hall: **New developments**

14:00-16:00 Emerging viruses & zoonoses II. (Main Hall)
(Chairs: **Norbert Nowotny** & **Stéphan Zientara**)

14:00-14:15 High prevalence of hepatitis E virus infection in pigs in different areas of the UK
Francesca Martelli, Sylvia S. Grierson, Alessandra Berto, Nadia Inglese, Catherine McCreary, Charlotte Dawson, Amanda Nevel, Malcolm Banks

14:15-14:30 Molecular epidemiology of hepatitis E virus in swine and wild boar in Germany
Edmilson F. Oliveira-Filho, Barbara R. Bank-Wolf, Heinz-Jürgen Thiel, Matthias König

14:30-14:45 Infection of domestic pigs with a human hepatitis E virus strain: symptoms, pathohistology and detection of viral RNA in different organs and tissues
Christine Bächlein, Frauke Seehusen, Sven Pischke, Heiner Wedemeyer, Wolfgang Baumgärtner, Beatrice Grummer

14:45-15:00 First discovery of HEV genotype 4 in swine in Europe through screening of fecal samples, the Netherlands and Belgium
Renate Hakze-van der Honing, Els van Coillie, Wim H. M. van der Poel

15:00-15:15 Phylogenetic analysis of partial and complete genome sequences of Hungarian hepatitis E virus strains with animal origin
Petra Forgách, Gábor Reuter, Helga Lussy, Norbert Nowotny, Tamás Bakonyi

15:15-15:30 Phylogenetic analysis of avian hepatitis E virus isolates from European and Australian chicken flocks supports the existence of three genotypes within avian HEV
Ivana Bilic, Ana Basic, Irina Prokofieva, Michael Hess

15:30-15:45 Discovery of new enteric viruses, noroviruses, in dogs
Vito Martella, Arianna Radogna, Eleonora Lorusso, Paschalina Moschidou, Krisztián Bányai, Gabriella Elia, Decaro Nicola, Costantina Desario, Canio Buonavoglia

15:45-16:00 Detection of contemporaneous human and bovine noroviruses in Belgium
Alexandra Scipioni, Axel Mauroy, Elisabeth Mathijs, Dominique Ziant, Georges Daube, Etienne Thiry

14:00-16:00 New developments (Lecture Hall)
(Chairs: **Véronique Jestin** & **Tamás Bakonyi)**

14:00-14:15 Different mechanisms contribute to RNA genome repair in the 5´ nontranslated region of bovine viral diarrhea virus
Stefanie Gilgenbach, Simone Preis, Paul Becher

14:15-14:30 Chemokine transcription is differently modulated in rainbow trout (O*ncorhynchus mykiss*) infected with viral haemorraghic septicaemia virus (VHSV) and infectious pancreatic virus (IPNV)
Jana Montero, Elena Chaves-Pozo , Alberto Cuesta , Carolina Tafalla

14:30-14:45 *In vitro* oncolytic capacities of two myxoma virus strains
Magalie Gretillat, Philippe Erbs, Jacqueline Gelfi, Christelle Camus, Stéphane Bertagnoli

14:45-15:00 Virus-host adaptation: whats new on myxomatosis?
Alexandra Muller, Eliane Silva, Joana Abrantes, Pedro J. Esteves, Paula G. Ferreira, Júlio C. Carvalheira, Norbert Nowotny, Gertrude Thompson

15:00-15:15 Myxoma virus as vaccine vector for new vaccination strategy against bluetongue
Sokunthea Top, Béatrice Pignolet, Eliane Foulon, Martine Deplanche, Stéphane Bertagnoli, Gilles Foucras, Gilles Meyer

15:15-15:30 Acid stability phenotypes in equine rhinitis B virus: serotype designation and recognition of dual infections
Jacquelyn Horsington, James Gilkerson, Nino Ficorilli, Michael Studdert, Carol Hartley

15:30-15:45 Developping a novel European surveillance system for detecting pathogens in wildlife: introducing WildTech
Richard G. Lea, Lisa Yon, Paul Barrow, Suzanne Boardman, Duncan Hannant

15:45-16:00 Epidemiology and control of classical swine fever in wild boar and potential use of a newly developed live marker vaccine
Frank Koenen, Andy Haegeman, Sándor Belák, Katinka Belák, Martin Beer, Matthias Kramer, Volker Moennig, Vittorio Guberti, Martin Hofmann, Juan Plana Duran, J. M. Sánchez-Vizcaíno, Gábor Kulcsár

16:00-18.00 **Poster session II:** Presenters of the posters in the topics listed below, are asked to be so kind as to stay at their poster: **Vaccines & viral immunology. Viral Diagnosis**.

16:30-17:00 *Coffee break*

17:00-18:00 **General Assembly of ESVV** (only for ESVV members)
(Scientific and financial report; admission of new members; election and/or reelection of board members; decision on the date & venue of the next international congress in 2012; miscellaneous topics)

19:30-22:30 *Gala Reception in the* **Hungarian Agricultural Museum (Vajdahunyad Castle)**

Celebration of the 20th anniversary of ESVV – with the support of

August 26 (Wednesday)

8:30-11:10 **Plenary Session** (Chairs: **Canio Buonavoglia** & **Ian H. Brown**)

8:30-9:10 Past present and future of EU funded research in animal health
Isabel Minguez-Tudela

9:10-9:50 Nothing in virology makes sense except in the light of evolution
Marian C. Horzinek

9:50-10:30 Microbe hunting in the 21st century
W. Ian Lipkin

10:30-11:10 Novel "swine-origin" H1N1 influenza in people: thoughts from a swine flu virologist
Kristien van Reeth

11:10-11:40 *Coffee break*

11:40-13:10 **Parallel Sessions**
Main Hall: **Evolution & genomics**
Lecture Hall: **Miscellaneous topics**

11:40-13:10 **Evolution & genomics** (Main Hall)
(Chairs: **Rachel Marschang** & **Béla Nagy**)

11:40-11:55 The genome of chicken parvovirus
László Zsák, J. Michael Day

11:55-12:10 Evidence of within-country clustering of canine parvoviruses from clinically-ill animals in mainland UK
Simon R. Clegg, Karen P. Coyne, Rosalind M. Gaskell, Susan Dawson, Alan D. Radford

12:10-12:25 The genome sequence of squirrel pox virus
Alan D. Radford, Colin J. McInnes, Alistair C. Darby, Neil Hall, Julian Chantrey

12:25-12:40 Phylogenetic analysis of PRRSV from Danish pigs
Charlotte K. Hjulsager, Solvej Ø. Breum, Lars E. Larsen

12:40-12:55 Comparative sequence analysis of a goose and a turkey aviadenovirus genome
Győző L. Kaján, Andrew J. Davison, Vilmos Palya, Balázs Harrach, Tibor Papp, Mária Benkő

12:55-13:10 Complete CSFV genome sequencing of recent CSFV isolates from Germany and comparison with sequences of former CSFV outbreaks
Immanuel Leifer, Bernd Hoffmann, Heinz-Günter Strebelow, Sandra Blome, Martin Beer

11:40-12:55 Miscellaneous topics
(Chairs: **Kristien Van Reeth** & **Tamás Tuboly**)

11:40-11:55 Preliminary report about the development of genomic and subgenomic (sg) Taqman real-time RT-PCR for detection and quantification of equine arteritis virus
Ákos Hornyák, Tamás Bakonyi, Norbert Nowotny, Miklós Rusvai

11:55-12:10 Pestivirus phylogeny: methods, strategies and discrepancies
Lihong Liu, Hongyan Xia, Claudia Baule, Sándor Belák, Niklas Wahlberg

12:10-12:25 Hepatitis E Virus is prevalent in the Danish pig population
Solvej Ø. Breum, Charlotte K. Hjulsager, Nilsa De Deus, Joaquim Segalés, Heléne Norder, Blenda Böttiger, Lars E. Larsen

12:25-12:40 Circulation of a BVD virus type 2 closely related to the North American hypervirulent viruses in Belgium
Carine Letellier, Bart Pardon, Piet Deprez

12:40-12:55 Antiviral activity of plant extracts against animal viruses
Viola Galligioni, Francesca Vaccari, Laura Gallina, Alessandra Scagliarini

13:15-13:25 **Closing Remarks**

13:30-15:30 *Farewell lunch*

15:30-17:00 Removal of posters

Poster presentations

Emerging viruses and zoonoses

Crimean-Congo hemorrhagic fever virus (CCHFV) and West Nile virus (WNV): molecular diagnosis and ticks survey in Turkey
Harun Albayrak, Emre Ozan, Mitat Kurt

Seroepidemiology of the arbovirus of Crimean-Congo hemorrhagic fever in rural community of Basra
Adel Alyabis, Hassan J. Hasony

Serosurvey of Italian dogs for exposure to influenza A viruses
Paola De Benedictis, Tara C. Anderson, Elisabetta Viale, Crispina Veggiato, Silvia Tiozzo Caenazzo, P. Cynda Crawford, Ilaria Capua

Inactivation of feline calicivirus and bacteriophages as models for hepatitis E virus in food matrices
Eva Emmoth, Frederik Widén, Irene Dergel, Sándor Belák

Tick-borne encephalitis outbreaks through raw milk consumption in Hungary
Emőke Ferenczi, Klára Széles, Zsuzsanna Balogh, Wlodzimierz Gut

Molecular analysis and characterization of swine and human influenza viruses isolated in Hungary in 2006-07
Péter Gyarmati, Giorgi Metreveli, Sándor Kecskeméti, Mónika Rózsa, Sándor Belák, István Kiss

Molecular detection of kobuviruses and recombinant noroviruses in cattle in continental Europe
Axel Mauroy, Alexandra Scipioni, Elisabeth Mathijs, Christine Thys, Etienne Thiry

Genetic characterisation of H9N2 viruses isolated from the Kingdom of Saudi Arabia
Isabella Monne, Alice Fusaro, Mahmoud M. Ismail, Mohamed H. Al-Blowi, Owais A. Khan, Annalisa Salviato, Alessia Schivo, Ilaria Capua, Giovanni Cattoli

Surveillance of emerging and/or neglected pathogens in pigeons in urban areas
Marta Pérez-Sancho, Julio Álvarez, Nerea García, Irene Martínez, Sergio González, Pilar Liébana, Joaquín Goyache, Francisco Mena, Lucas Domínguez

Design of an ELISA test specific for genotype 3 hepatitis virus E in swine
Nicolas Rose, Annie Boutrouille, Audrey Fraisse, Aurelie Lunazzi, Marc Eloit, Nicole Pavio

Four different sublineages of highly pathogenic avian influenza H5N1 introduced in Hungary in 2006-2007
Zsófia Szeleczky, Ádám Dán, Krisztina Ursu, Éva Ivanics, István Kiss, Károly Erdélyi, Sándor Belák, Claude P. Muller, Ian H. Brown, Ádám Bálint

Epizootiology

Investigating the presence of infectious haematopoietic necrosis (IHN) and infectious pancreatic necrosis (IPN) virus infections in rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792)
Harun Albayrak, Emre Ozan, Yuksel Durmaz

Respiratory disease associated with bovine coronavirus in adult cattle in Northern Portugal
Ana Cristina Araújo, Eliane Silva, Alexandra Muller, Gertrude Thompson

First Austrian case of hepatitis contagiosa canis in over 20 years in a dog imported from Hungary
Viviane Benetka, Ingrid Gutlederer, Muna Latif, Karin Walk, Karin Möstl

Molecular epidemiology of bovine coronavirus in Sweden
Mehdi R. M. Bidokhti, Anna Ohlson, Madeleine Tråvén, Stefan Alenius, Mikhayil Hakhverdyan, Sándor Belák

BoHV-4 infection in dairy cattle with reproductive disorders in Turkey
Seval Bilge Dağalp, Alireza Faraji Majarashin, Tuba Çiğdem Oğuzoğlu, Elvin Çalişkan, Başak Altaş, Dilek Muz, Feray Alkan

Further indications to the vertical transmissibility of goose haemorrhagic polyomavirus
Attila Farsang, Sándor Bernáth, Mihály Dobos-Kovács

Effect of PCV-2 on the swine transcriptome *in vivo*
Béatrice Grasland, Yannick Blanchard, Véronique Béven, Hélène Félix, Anne-Cécile Nignol, Roland Cariolet, André Jestin

Phylogenetic and antigenic characterisation of chicken astroviruses – identification of a new, genetically and serologically distinct group
Tamás Mató, Edit Fodor, Tímea Tatár-Kis, Vilmos Palya

Evolution and genomics

The genomic constellation of a novel avian orthoreovirus strain associated with runting stunting syndrome
Krisztián Bányai, John K. Rosenberger, Tamás Mató, Vilmos Palya

Genetic characterization of canine distemper virus (CDV) from dogs and red foxes in Greece
Charalambos Billinis

Entire genomic characterisation of 10 low pathogenic H5 subtype avian influenza viruses collected in France between 2002 and 2008
François-Xavier Briand, Ghislaine Le Gall-Reculé, Katell Ogor, Cécile Guillou-Cloarec, Aurélie Bluteau, Céline Macé, Séverine Ferré, Audrey Schmitz, Pascale Massin, Olivier Guionie, Josiane Lamandé, Chantal Allée, Martine Cherbonnel, Jean-Paul Picault, Véronique Jestin

Adaptation of avian influenza viruses (subtype H9) to different avian hosts
Meike Diederichs, Maren Bohm, Henning Petersen, Christine Winter, Silke Rautenschlein, Christel Schwegmann-Wessels, Georg Herrler

Genome analysis of fish adenovirus seems to point to a need of establishing the fifth adenovirus genus
Andor Doszpoly, Balázs Harrach, Mária Benkő

Preliminary studies in the development of a minor-groove binder PCR assay for the phylogenetic characterisation of PCV2 isolates
Catherine Duffy, Julie McClintock, Michael McMenamy, John McKillen, Gordon M. Allan

Genetic diversity of bovine viral diarrhoea virus isolates from Italy
Monica Giammarioli, Elena Canelli, Sara Ciulli, Elisabetta Rossi, Gian Mario De Mia

Genetic and antigenic characterization of a novel pestivirus genotype
Monica Giammarioli, Severina Anna La Rocca, Cristina Casciari, Elisabetta Rossi, Falko Steinbach, Gian Mario De Mia

Phylogenetic analysis of porcine reproductive and respiratory syndrome virus isolates from Slovakia
Anna Jackova, Michaela Novackova, Stefan Vilcek

Characterisation of a non-protective and a non-pathogenic infectious rabbit haemorrhagic disease-like virus
Ghislaine Le Gall-Reculé, Françoise Zwingelstein, Marie Fages, Stéphane Bertagnoli, Jacqueline Gelfi, Stéphane Marchandeau

Detection of porcine circovirus in rodents
Márta Lőrincz, Attila Cságola, Imre Biksi, Levente Szeredi, Ádám Dán, Tamás Tuboly

Molecular analysis of equine herpesvirus 1 strains isolated in the last 30 years in Hungary
Péter Malik, Ádám Bálint, Ádám Dán, Vilmos Pálfi

Incidence of porcine circovirus type 2 (PCV2) and Torque teno virus (TTV) genogroups in pigs from post-weaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected farms in archival UK samples
Michael J. McMenamy, Irene McNair, Catherine Duffy, John McKillen, Nuala Fee, Catherine Mulholland, Brian Adair, Gordon Allan

Molecular characterization of canine parvovirus type 2 circulating in Greece
Vasileios Ntafis, Efthychia Xylouri, Iris Kalli, Katerina Adamama-Moraitou, Timoleon Rallis, Theofanis Kanellos, Costantina Desario, Nicola Decaro

Molecular epidemiology of rabies virus isolates in Slovenia
Danijela Rihtarič, Ivan Toplak, Urška Kuhar, Jože Grom, Peter Hostnik

Phylogenetic analysis of small-ruminant lentiviruses in sheep flocks / goat herds in Greece
Vassiliki Spyrou, Vassilios Psychas, Georgios Sofianidis, Vasia Mavrogianni, Emmanouil Kalaitzakis, George Fthenakis, Charalambos Billinis

First description of swine Torque teno virus (TTV) and detection of a new genogroup in Hungary
Mária Takács, Ágnes Dencs, Csenge Csiszár, Andrea Hettmann, Erzsébet Rusvai, Katalin N. Szomor, Vilmos Pálfi, Béla Nagy

Characterisation of the murine adenovirus 2 genome and partial sequences from similar rodent adenoviruses
Márton Vidovszky, Sandra Ramelli, Willy Decurtins, Justyna Ruminska, Andor Doszpoly, Gabriella Skoda, Máté Jánoska, Balázs Harrach, Urs Greber, Silvio Hemmi

Host-virus interactions and pathogenesis

An infectious clone of West Nile virus IS-98-ST1
Céline Bahuon, Sylvie Lecollinet, Eva Mertens, Stéphan Zientara, Philippe Despres

Comparison of the long fiber of serotype 1 fowl adenovirus isolates from chickens with gizzard erosions
Ana Basic, Evelyn Schulz and Michael Hess

Comparative immunohistochemical studies on the tissue distribution and persistency of three variants of classical swine fever virus in experimentally infected pigs: a recent isolate of wild boar origin, a newly developed modified live marker vaccine and the conventional “C” vaccine strain
Katinka Belák, Marylène Tignon, Gábor Kulcsár, Tímea Barna, Katalin Fábián, Réka Lévai, Attila Farsang, Frank Koenen, Sándor Belák

Avian influenza virus (AIV) pathogenesis in a ferret model: H5N1 clade 2.2 (A/turkey/Turkey/1/05)
Sharon M. Brookes, Fanny Garcon, Dianne Major, Lorenzo Rapisarda, Alejandro Nunez, John Wood, Ian H. Brown

Characterization of *in vitro* parameters correlating with virulence of classical swine fever virus in pigs
Rita Eymann-Haeni, Artur Summerfield, Sandra Blome, Irene Greiser-Wilke, Nicolas Ruggli

Modulation of cytokines and interferon type 1 pathway by African swine fever virus of different virulence, ASFV/L60 and ASFV/NH/P68
Solange Gil, Nuno Sepúlveda, Emmanuel Albina, Alexandre Leitão, Carlos Martins

Development of strand-specific quantitative RT-PCR assay for bovine viral diarrhoea virus replication
S. Anna La Rocca, Rachelle Cook, Torstein Sandvik, Rebecca M. Strong

Cytokine gene expression profiling after infection of porcine cells with classical swine fever virus using SAGE and RT-qPCR
Anastasia Lange, Irene Greiser-Wilke, Stefanie Bendfeldt, Laura C. Miller, John D. Neill

The effect of infection with feline calicivirus on host cytoskeletal proteins
Emma L. Newsham, Alan D. Radford, David G. Spiller, Susan Dawson, Rosalind M. Gaskell, Jonathan M. Wastling

Role of CD9 for the entry of bovine viral diarrhea virus into the cell
Katrin Pannhorst, Stefanie Bendfeldt, Inga Grotha, Beatrice Grummer, Irene Greiser-Wilke

Analysis of well-differentiated porcine airway epithelial cells as host cells for swine influenza viruses
Darsaniya Punyadarsaniya, Isabel Hennig-Pauka, Christine Winter, Christel Schwegmann-Wessels, Georg Herrler

Interferon involvement in borna disease virus infection
Jonas J. Wensman, Anne-Lie Blomström, Carolina Ilbäck, Elina Sandwall, Anders Johannisson, Sándor Belák, Anna-Lena Berg, Mikael Berg

Vaccines and viral immunology

Evaluation of two modified live CSFV marker vaccines in a comparative animal trial
Andrea Aebischer, Martin Hofmann

Targeting to antigen-presenting-cells by DNA vaccination induces protective responses against foot-and-mouth disease in swine
Belén Borrego, Jordi M. Argilaguet, Eva Pérez-Martín, Mariano Pérez-Filgueira, José M. Escribano, Francisco Sobrino, Fernando Rodríguez

Compared cross-immunogenicity of different avian influenza H5 hemagglutinins using a DNA vaccination model in Muscovy ducks
Olivier Guionie, Marie-Odile Le Bras, Stéphanie Bougeard, Michel Bublot, Véronique Jestin

Putative antigenic domains on the E^{rns} protein of pestiviruses
Denise Henrych, Volker Moennig, Irene Greiser-Wilke

Antigenic variation in erboviruses
Jacquelyn Horsington, James Gilkerson, Carol Hartley

Improved tools and strategies for the prevention and control of classical swine fever (CSFV_goDIVA)
Frank Koenen, Andy Haegeman, Åse Uttenthal, Marie-Frédérique Le Potier, Gábor Kulcsár, Willie Loeffen, Juan Plana Durán, Martin Beer, Gian Mario De Mia, Sophie Rossi, Rong Gao, Sándor Belák, Volker Moennig, Jose Sanchez-Vizcaino, Hans-Hermann Thulke, Niels Delater, Paolo Calistri, Martin Hofmann

In vitro and *in vivo* characterisaton of bovine viral diarrhea virus (BVDV) N^{pro} deletion mutants
Patricia König, Johanna Zemke, Katrin Mischkale, Ilona Reimann, Martin Beer

Protective efficacy of FMDV subunit vaccine produced using a silkworm-baculovirus expression system against two Chinese type Asia I isolates in cattle
Zhiyong Li, Xiangping Yin, Yongzhu Yi, Baoyu Li, Xuerui Li, Xi Lan, Zhifang Zhang, Jixing Liu

Importance of individual neutralising epitopes of foot-and-mouth disease virus in relation to polyclonal response of vaccinated animals
Mana Mahapatra, Pip Hamblin, David Paton

Alternative testing of viral vaccines for extraneous agents: detection of egg drop syndrome virus by PCR
Enikő Petrovszki, Tibor Farkas, Erzsébet Sombor, Zoltán Pénzes

Early protection against lethal CSFV challenge infection after immunization of pigs with the modified live marker vaccine candidate CP7_E2alf
Ilona Reimann, Immanuel Leifer, Elke Lange, Sandra Blome, Sandra Juanola, Joan Plana Duran, Martin Beer

Coexpression of rabies virus glycoprotein and nucleoprotein in silkworm-baculovirus expression system and its utilization as a subunit vaccine
Xiangping Yin, Zhiyong Li, Yinv Li Jiangtao Li, Yongzhu Yi, Huiguo Shu, Zhifang Zhang,Jixing Liu

Viral diagnosis

Immunocapture ELISA and RT-PCR in the diagnosis of PPRV infection
Harun Albayrak, Feray Alkan

Detection of hepatitis E virus-specific IgG antibodies in domestic pigs from Germany using a novel in-house ELISA
Christine Bächlein, Anika Schielke, Reimar Johne, Beatrice Grummer

Potential risks for the vertical transmission of bovine viral diarrhoea (BVD) virus
Jelena Balatinec, Tomislav Keros, Mirko Lojkić, Lorena Jemeršić, Ivana Lojkić, Željko Cvetnić, Tomislav Bedeković

Detection of a novel porcine boca-like virus in the background of porcine circovirus type 2 induced postweaning multisystemic wasting syndrome
Anne-Lie Blomström, Sandor Belák, Caroline Fossum, John McKillen, Gordon Allan, Per Wallgren, Mikael Berg

Field evaluation of a bulk milk monitoring programme of bluetongue virus 8 (BTV-8) infection in a bluetongue free area
Giulio Bucci, Ernst Stifter, Lebana Bonfanti, Laura Gagliazzo, Stefano Nardelli

Developement of a novel real time RT-PCR for the detection of the NS2A gene of West Nile virus (WNV)
Antonella Cersini, Ilaria Maria Ciabatti, Armando Damiani, Giuseppe Manna, Maria Teresa Scicluna, Gian Luca Autorino

Phylogenetic analysis of the hexon gene of avian adenovirus from commercial chickens and turkeys
Jorge L. Chacón, Antonio C. Pedroso, Juliana Rodriguez, Camila Peloso, Joelma Moura, Claudete Ferreira, Antonio P. Ferreira

Evaluation of the CACO-2 cell line for isolation of swine influenza virus compared to standard methods
Chiara Chiapponi, Irene Zanni, Chiara Garbarino, Giuseppe Barigazzi and Emanuela Foni

A commercially available in-house test is able to detect the new variant 2C of canine parvovirus
Nicola Decaro, Costantina Desario, Melissa J. Beall, Maria Loredana Colaianni, Anthony A. DiMarco, Francesca Amorisco, Alessandra Cavalli, Canio Buonavoglia

Validation of developed AIV real-time RT-PCR assays through participation in interlaboratory comparison tests
Maia Elizalde, Maria Yuste, Marisa Arias, Jovita Fernández-Pinero

First detection and partial characterization of koi herpesvirus (KHV) in Slovenia
Aleksandra Grilc Fajfar, Ivan Toplak, Peter Hostnik, Vlasta Jenčič

Enzootic bovine leukosis: what indications are suitable to standardize and validate the milk ELISA test for indirect diagnosis?
Francesco Feliziani, Francesco Vitelli, Francesco Cesarini, Antonio De Giuseppe

Detection of neuropathogenic strains of equine herpesvirus 1 (EHV-1) associated with abortion in Germany
Ann-Kathrin Fritsche, Kerstin Borchers

Detection of bovine viral diarrhoea virus (BVDV) in bovine abortions
David A Graham, Norman Beggs, Karen Mawhinney, Maria P. Guelbenzu, Vi Calvert, Ben Cunningham, Lynsey Rowan-Layberry, Ian McLaren

Validation of a competitive ELISA for serodiagnosis of PRRS based on recombinant N-protein and monoclonal antibody
Santina Grazioli, Giulia Pezzoni, Paolo Cordioli, Emiliana Brocchi

Epidemiological survey of pathogens associated with porcine respiratory diseases in pigs from North-Western Germany from 2007 to 2009
Beatrice Grummer, Ludwig Haas, Elisabeth Grosse Beilage, Heiko Nathues, Irene Greiser-Wilke

Comparison of four real-time reverse transcription polymerase chain reaction assays for the detection of bovine viral diarrhoea virus nucleic acid
Maria Guelbenzu, David Graham

Detection of ASFV using LATE-PCR and rotary design amplification system
Mikhayil Hakhverdyan, Bonnie Ronish, Carmina Gallardo, Jovita Fernández-Pinero, Karl Ståhl, Neil LeBlanc, Lawrence Wangh, Sándor Belák

ELISA for detection of avian influenza H5 and H7 antibodies
Trine H. Jensen, Gitte Ajjouri, Kurt Handbjerg, Marek Slomka, Vivien Coward, Martine Cherbonnel, Poul H. Jørgensen

VHS outbreaks in Finland 2000-2008
Sirkka-Liisa Korpenfelt, Tiina Nokireki, Ilona Laamanen, Tuija Gadd

Lateral flow technology – for a quick on-site differentiation of FMDV and other vesicular diseases
Therese Kristersson, Ann Nordengrahn, Malik Merza

Potential DIVA diagnostics for avian influenza: a comparison and validation study
Katrin Kühn, Martin Beer

Development of a real-time PCR asssay based on primer-probe energy transfer (PriProET) for the sensitive dectection of infectious laryngotracheitis virus
Michael J. McMenamy, John McKillen, Bernt Hjertner, Brian Adair, Gordon Allan

Development of an accelerated loop-mediated isothermal amplification (LAMP) assay for sensitive dectection of infectious laryngotracheitis virus
Michael J. McMenamy, John McKillen, Bernt Hjertner, Brian Adair, Gordon Allan

Towards a Jembrana disease virus specific diagnostic immunoassay – peptide mapping of Gag and Env proteins of bovine lentiviruses
Tegan J. McNab, Moira Desport, I. W. Masa Tenaya, Nining Hartaningsih, Graham E. Wilcox

Genetic characterization of chicken parvovirus strains from naturally infected Hungarian flocks
Elena Alina Palade, Ákos Hornyák, Zoltán Demeter, Mihály Dobos-Kovács, Zsófia Benyeda, Miklós Rusvai, János Kisary

A baculovirus expressed NS3 protein of bovine viral diarrhea virus displays conformational and antigenical properties as the native protein
Giulia Pezzoni, Emiliana Brocchi

First evidence of caprine herpesvirus 1 in goat herds in Piedmont, Italy
Monica Pitti, Antonia Sciarra, Mariangela Andrà, Pasquale Amato, Giuseppe Ru, Loretta Masoero

Pyrosequencing analysis for a rapid fowl adenovirus species classification
Matteo Samuele Pizzuto, Cristian De Battisti, Sabrina Marciano, Ilaria Capua, Giovanni Cattoli

Development of a competive ELISA (C-ELISA) system for the detection of avian influenza infection in sera of five different avian species
Michela Rigoni, Elisabetta Viale, Marzia Mancin, William Dundon, Valeria Brasola, Calogero Terregino, Ilaria Capua

Genetic characterization of coronaviruses in shelter dogs and cats in Lisbon
Ricardo C. Rosado, Ana Duarte, Augusto Baptista, Filomena Oliveira, Ana Machado, Leonel Fernandes, Luís Tavares

Virological survey in shelter dogs and cats in Lisbon
Ricardo C. Rosado, Ana Duarte, Augusto Baptista, Filomena Oliveira, Ana Machado, Leonel Fernandes, Luís Tavares

Interlaboratory validation of real-time PCR assay for detection of African swine fever virus
Marylène Tignon, Carmina Gallardo, Marie-Frédérique Le Potier, Raquel Nieto, Elena Martín, Virginia Pelayo, Evelyne Hutet, Yves Van der Stede, Marisa Arias, Frank Koenen

Avian nephritis virus commonly detected by RT-PCR in field and longitudinal samples from broiler flocks
Daniel Todd, Nigel McBride, James Trudgett, Brendan Donnelly, Francis McNeilly, Victoria J. Smyth, Heather L. Jewhurst, Brian M. Adair

Seroprevalence of chicken astrovirus infections
Daniel Todd, Donna L. Wilkinson, Mildred Wylie, Heather L. Jewhurst, Brian M. Adair

Validation of a real-time RT-PCR for the detection of bluetongue virus in bull semen and development of an internal control
Tine Vanbinst, Frank Vandenbussche, Kris De Clercq

Description and validation of four real-time RT-PCR for the serotyping of BTV-1, BTV-6, BTV-8 and BTV-11
Frank Vandenbussche, Ilse De Leeuw, Kris De Clercq

Proximity ligation assay (PLA) is a new method for the detection of avian viruses
Alia Yacoub, Joerg Schlingemann, Mikael Leijon Istvan Kiss, Siamak Zohari, Anna Matyi-Tóth, Ann Nordengrahn, Ulf Landegren, Bjorn Ekstrom, Sándor Belák

Viruses of wild and exotic animals

Investigation of avian influenza virus (AIV) in wild birds in Turkey
Harun Albayrak, Emre Ozan

First investigation of West Nile (WNV) in wild birds in Turkey
Harun Albayrak, Emre Ozan

Long-term monitoring of wild birds for ortho- and paramyxoviruses in North-Western Italy
Rosalia Centorbi, Francesca Rizzo, Riccardo Orusa, Elisa Barcucci, Maria Lucia Mandola

PCR screening of mammalian predators (Carnivora) for adeno- and herpesviruses
Eszter Dandár, Andor Doszpoly, Máté Jánoska, Miklós Heltai, László Szabó, Mária Benkő

Feasability study of a capture and testing system for wild birds to set up an ”early warning system” for West Nile virus in horse and bird in Belgium
Joëlle De Vriese, Olivier Poncin, Bénédicte Lambrecht, Didier Vangeluwe, Thierry van den Berg, Carine Letellier

Reovirus related pathological lesions and consequential death in rough green snake (*Opheodris aestivus*)
János Gál, Míra Mándoki, Miklós Rusvai, Judit Tavaszi, Szilvia Farkas

Novel simian adenoviruses – comparison with formerly isolated primate adenoviruses
Máté Jánoska, Andor Doszpoly, Győző Kaján, Laura Pantó, Balázs Harrach

Complete sequence and genetic features of raptor adenovirus 1: a novel, non-isolated species in the genus *Siadenovirus*
Endre Kovács, Balázs Harrach, Mária Benkő

Phylogenetic characterisation of canine distemper viruses detected in naturally infected wild and domestic carnivores in North East of Italy
Isabella Monne, Annalisa Salviato, Carlo Citterio, Mariapia Cova, Marco Bregoli, Paola De Benedictis, Claudio Pasolli, Elena Mazzolini, Viviana Valastro, Giovanni Farina, Karin Trevisiol, Giovanni Cattoli

Invertebrate iridovirus infections in insectivorous pets? Quest for a better diagnostic method
Tibor Papp, Dirk Spann, Rachel E. Marschang

Examinations aiming at the verification of the reptilian origin of atadenoviruses
Judit Pénzes, Andor Doszpoly, Balázs Harrach, Mária Benkő

Real time RT-PCR assays for monitoring avian influenza A H5/H7 viruses in wild and domestic birds
Francesca Rizzo, Francesca Sidoti, Cristina Costa, Massimiliano Bergallo, Rossana Cavallo, Maria Lucia Mandola

Detection of bluetongue serotype 4 in wax-embedded tissues from wild ruminants in the South of Spain
Belén Rodríguez-Sánchez, Pedro J. Sánchez-Cordón, Verónica Molina, María A. Risalde, Ana Cristina Pérez de Diego, José C. Gómez-Villamandos, José M. Sánchez-Vizcaíno

Genome sequence of a border disease virus strain isolated from a Pyrenean chamois
Stefan Vilček, Kim Willoughby, Peter F. Nettleton, Peter Becher

Search for hepatitis E virus in wild life reservoirs and characterization of the virus in Swedish wild boars by full length sequencing
Frederik Widén, Anna Matyi-Toth, Heléne Norder, Sándor Belák

Plenary lectures

VETERINARY VIROLOGY IN THE REAL WORLD

Steven EDWARDS

Visiting Professor of Veterinary Pathology, University of Liverpool, UK

Virology is a relatively young discipline. With all due respect to the 18th and 19th century pioneers such as Jenner and Pasteur who made significant progress in controlling diseases that we now know are caused by viruses, the science of virology is largely a 20th century phenomenon. Virological history in that time relates a succession of fresh discoveries about those mysterious, invisible but filterable agents that nevertheless seemed to be linked to some of the world's most devastating epidemics, beginning of course with the first discovery of a vertebrate virus (foot and mouth disease virus) by Loeffler and co-workers. We are fortunate therefore to have known and worked alongside those, like Dinter, whose experience takes us back to the earliest and perhaps most exciting days of this discipline. Veterinary virology has developed in parallel with medical virology, benefiting from the greater investment in research and technology although the vets have the advantage of conducting experimental work in their subjects' natural hosts. Nor does virology exist as a stand-alone science. The expansion of immunology and of cellular biology at the molecular level have in turn revolutionised our understanding of the nature of viruses and their interactions with the host cell. Dinter's professional life spanned an enormous transformation in virological techniques, from the application of cell cultures, ultramicroscopy, ever more sophisticated immunoassays, through to the early days of the molecular revolution. This enables us to move away from dependency on virus propagation and opens up prospects of astonishingly sophisticated diagnostic tools and highly effective vaccines. Combined with the expansion of data analytical tools brought by ever-expanding computer power, including the related disciplines of epidemiology and disease modelling, virology in the 21st century will soon be unrecognisable to those early pioneers. Looking beyond the molecule even to subatomic events, we could speculate whether the astonishing and (to a mere biologist) baffling developments currently happening in particle physics and quantum theory may come to have an impact on virological science in a future and uncertain world.

Twenty years ago some of us had the idea to establish a European Society for Veterinary Virology. We had to feel our way, to take soundings from those involved in the discipline, not all of whom were supportive, and to set a direction that balanced the pursuit of basic scientific understanding of viruses and their interactions with the host, with practical applications that could lead to improved diagnosis and disease control. We also faced practical issues such as how to fund the organisation as it had no corporate allegiance, being run by scientists for scientists. Central administration costs have been kept minimal, thanks to the support of the parent institutions of the successive committee members and officers, and early on we set the target that each scientific meeting should be self sustaining through a combination of registration fees and sponsorship. Without at this point naming names I should acknowledge the tremendous support for ESVV by our various sponsors, without which none of it would have been possible. We went ahead with the first Congress in 1989 in Liège, when Zvonimir Dinter spoke on the topic of "Why a European society for veterinary virology?" (Vet Microb. 23: 8-10, [1990]) He emphasised from both past experience and future needs the importance of fostering a dialogue between East and West in Europe, using the common goals of veterinary virology to overcome cultural and linguistic barriers and produce solutions to the challenges presented by virus diseases of animals. Twenty years on the challenges have changed, new solutions have emerged, but disease problems remain. From that somewhat tentative but also confident beginning the Society has gone from strength to strength and looking at the programme for this current congress we can be assured that veterinary virology in Europe is strong and looking forward to a successful future.

BIODIVERSITY OF MAMMALS, BIRDS, AND VIRUSES

Paul-Pierre PASTORET

Head of the Publications Department, World Organisation for Animal Health (OIE), 12, rue de Prony, 75017 PARIS, France

Biodiversity is a key element in the emergence of infections in domestic animals and humans. There are several mechanisms of emergence: they include globalisation, climate changes and the opening of previously closed ecosystems. The variability of pathogens is also an important factor in the emergence of disease, especially with regard to viruses (particularly the highly variable RNA viruses), as it leads to populations of quasi-species. Approximately 75% of all the recently emerging diseases originate in wildlife. Wildlife obviously constitutes an important potential reservoir for new disease agents that are pathogenic to humans and domestic animals.

The number of viruses already identified is approximately 5,000, but the actual number could exceed 200,000, according to the first estimates. Even this number is most likely underestimated. If one considers that there are 5,416 recognised mammal species, and that, for instance, *Herpesviruses* have been isolated from all classes of vertebrates and even from oysters, it becomes clear that the world of viruses is huge and constantly evolving.

The expected number of mammalian species is estimated to be around 5,500, and as 5,146 (99%) of these species have already been identified, we are nearly at the end of the inventory.
Among mammals, there are 2,277 rodent species pertaining to 481 genera. Rodents compose, therefore, 42% of recognised mammal species. This number is particularly important if one takes into account the fact that the order of rodents harbours, and is the reservoir of, numerous zoonotic infections. To date, the order of *Chiroptera* contains 1,116 species, pertaining to 202 genera. Bats, therefore, make up 20.6% of the total number of mammal species; another worrying potential source of emerging infection.

There are approximately 10,000 species of birds, pertaining to 2,058 genera; the number of passerines being 5,712.

In order to take appropriate measures to control infection in wildlife, domestic animals and humans, it is important to consider the epizootiological/epidemiological role played by the different wildlife species as the reservoir of an infection of domestic animals and, conversely, the role of domestic animals as a reservoir of wildlife infection. For example, it was possible to eradicate smallpox in humans mainly due to the fact that human beings were the only reservoir of the infection. A similar thing could be true for rinderpest in cattle, since wildlife seems to be more an epidemiological dead-end than a reservoir. In addition, the elimination of an infection at its animal source can result in the elimination of the infection in other target species (such as human beings), as was clearly seen in the elimination of terrestrial rabies in Western Europe.
When dealing with biodiversity, one must also consider the biodiversity of domestic species, with many domestic breeds differing in their susceptibility to different infections.

Viruses are often simply seen as a source of infection in humans and animals; the positive aspects of their interaction with their hosts at both population or individual level is rarely appreciated/acknowledged. We should look more deeply at their contribution to the evolutionary pattern of vertebrates/invertebrates, even if the mutation rate of viruses is much higher than that of their hosts.

All these facts have led to the recent concept of “One World, One Health”.
In 2008, four international organisations, FAO (United Nations Food and Agriculture Organization), the World Organisation for Animal Health (OIE), the World Health Organization (WHO) and UNICEF (United Nations Children’s Fund), along with the World Bank and UNSIC (United Nations System Influenza Coordinator), joined forces to produce a strategic document entitled ‘Contributing to One World, One Health[1]: a strategic Framework for Reducing Risks of Infectious Diseases at the Animal–Human–Ecosystems Interface’.

The document stems from a meeting held in Manhattan (New York, United States of America) in September 2004, bringing together experts in various disciplines from around the world to discuss problems arising from the circulation of diseases between humans, domestic animals and wildlife. The product of this first meeting on the subject of these three domains was the formulation of the Manhattan Principles. These twelve principles seek to define a holistic approach to the prevention of epidemic/epizootic diseases, while maintaining the integrity of ecosystems for the benefit of mankind, our domestic animals and biodiversity, a topic that concerns us all.
Mankind is currently facing many different challenges, which will require global solutions. One of these challenges is the spread of infectious diseases that emerge or re-emerge at the interfaces between animals, humans and the ecosystems in which they live. This situation is the result of several factors, including the exponential growth in human and livestock populations, rapid urbanisation, changing farming systems, closer interaction between livestock and wildlife, forest encroachment, changes in ecosystems and globalisation of trade in animals and animal products.
The most important factor is undoubtedly the dramatic increase in the world’s population, which is expected to reach 8 billion by 2025, mainly in Asia, Africa and Latin America.
At the same time, some in-transition Asian countries are currently experiencing strong economic growth, with rapid urbanisation and greater demand for food, particularly of animal origin. Termed the “livestock revolution” by Delgado, this phenomenon is leading to rapid change in farming systems. In 2008, over 21 billion food animals were produced to help feed a population of over 6 billion people. By 2020, this demand is expected to increase by 50%.
The increase in the human population is also putting pressure on land use, with further encroachment on natural forests and their rich and diverse fauna, thereby exposing humans and domestic animals to new pathogens.
The overarching objective of the strategic framework proposed in the document “One World, One Health” is to minimise the global impact of diseases of animal origin, including zoonoses, especially those with pandemic potential.

[1] One world, one health is still a concept created by the Wildlife Conservation Society

A LIVE ATTENUATED VACCINE AGAINST FELINE INFECTIOUS PERITONITIS (FIP) PROTECTING BY CELLULAR RATHER THAN VIRUS-NEUTRALIZING IMMUNITY

Bert Jan HAIJEMA, Pepijn SCHELLEN, Herman EGBERINK, Peter J. M. ROTTIER

Virology Division, Faculty of Veterinary Sciences, Utrecht University, The Netherlands

Keywords: coronavirus, feline infectious peritonitis, protective immunity, vaccine

Introduction and objectives

Feline infectious peritonitis (FIP) is a fatal disease of cats caused by a coronavirus, the feline infectious peritonitis virus (FIPV). Currently, the virus is the leading infectious cause of mortality in young cats in pedigree catteries and shelters. Because an effective vaccine is not available, our aim was to develop one.

A major problem in feline coronavirus (FCoV) research is that the viruses can not be grown in vitro. An exception are the rare variants that occasionally emerge as a result of recombination between FCoVs and canine coronaviruses (CCoVs) during co-infection of animals by these viruses in the field. These so-called serotype II FCoVs invariably have acquired a CCoV spike gene (+ some flanking sequences), explaining their altered growth properties. Due to the large differences between FCoV and CCoV spikes, antisera to the 'true' (serotype I) and the serotype II FCoVs do not cross-neutralize. Yet, because cell-mediated immunity has been suggested to contribute to immunity against FIPV, and in order to obviate the well-known complicating risk of inducing immune-enhancing antibodies, we based our vaccine approach on the virulent serotype II strain FIPV 79-1146.

Materials and methods

Using targeted RNA recombination we rationally designed attenuated viruses by deletion of genes implicated in viral virulence.

Results and conclusions

We showed that oronasal vaccination with these deletion viruses can protect cats against FIP when challenged with homologous (i.e. serotype II) virulent FIPV. Using one of these vaccine candidates (FIPVΔ3abc) we subsequently observed excellent protection also upon challenge with serotype I FIPV, the predominant serotype in the field. To further attenuate the vaccine and thereby improve its safety, the order of the structural protein genes in FIPVΔ3abc was rearranged. The resulting virus appeared to be viable and to grow well, while the excellent protective capacity against serotype I virus challenge had remained unaltered. This protection was unlikely to be mediated by antibodies as the sera from the vaccinated animals did not neutralize serotype I FIPV in vitro. The observations are consistent with the earlier indications that cellular rather than humoral immunity is essential for protection. While protection solely based on virus neutralizing antibodies has been reported in numerous vaccination studies, protection not involving virus neutralization, as observed here, has - to our knowledge - not been demonstrated earlier. The vaccine candidate thus obtained is ready to be tested in the field and, if successful, to be prepared for the market.

AN EDUCATED GUESS ON THE PATHOGENIC FORCES BEHIND SHEEP-ASSOCIATED MALIGNANT CATARRHAL FEVER

Mathias ACKERMANN, Claudia MEIER-TRUMMER

Institute of Virology, University of Zurich, Winterthurerstrasse 266a, 8057 Zurich, Switzerland

Malignant catarrhal fever (MCF) is a lethal disease of various cloven-hoofed animals. Several members of the genus Macavirus within the subfamily *gammaherpesvirinae* are considered as causative agents of MCF. Prominently, the alcelaphine herpesvirus 1 (AlHV-1) is considered as the prototype of wildebeest-derived MCF (WD-MCF), whereas the ovine herpesvirus 2 (OvHV-2) has been recognized as the major agent of sheep-associated MCF (SA-MCF).

Importantly, these viruses cause no apparent disease in their natural ruminant host species, whereas they trigger disease characterized by vasculitis, necrosis, and accumulation of activated, dysregulated cytotoxic lymphocytes in various tissues of animals, which are susceptible to MCF. However, the pathogenic basis of the disease remains mysterious.

Genetically, the two viruses are quite similar, sharing in the majority of genes homology with genes of other gammaherpesviruses. However, each one has a set of genes that distinguishes them from the other gammaherpesviruses. Within this set, there are eight genes that are present in both viruses, whereas two additional genes (A1, A4) are unique to AlHV-1 and three (Ov2.5, Ov3.5, Ov4.5) are unique to OvHV-2. Several members among this set of distinguishing genes have been predicted to be involved into major processes, such as transcriptional regulation or intra- and inter-cellular signalling. Indeed, it seemed likely that those genes and their products may be involved in the pathogenesis of MCF.

Based on those assumptions, we hypothesized that the gene expression patterns of OvHV-2 and the relative abundances of host cell transcripts in lymphnodes may be used to identify pathways that may help to explain the pathogenesis of MCF. Therefore, viral and host cell gene expression patterns in lymph nodes of animals with MCF and healthy controls were analyzed by microarray. Two regions on the viral genome were transcriptionally active, one encoding an orthologue to the latency-associated nuclear antigen (ORF73) of other gamma herpesviruses, the other with no predicted open reading frame. Interestingly, this OvHV-2 gene expression profile is similar to the viral gene expression activity found in the context of AlHV-1-associated MCF in the rabbit model described by others. Thus, the major common observation was that silence, rather than activity of viral genes was associated with MCF.

On the host's side, a vast number of transcripts related to inflammatory processes, lymphocyte activation, cell proliferation and apoptosis were detected at different abundances in animals with SA-MCF. However, the IL-2 transcript was eminent among the transcripts, which were, compared to healthy controls, less abundant in animals with SA-MCF. Similarly, a loss of CD4-positive lymphocytes, which are the major IL-2-producing cells, was observed by others in rabbits with WD-MCF.

Since the phenotype of humans and mice with deficient IL-2-system almost perfectly matches the disease signs observed in cattle with MCF, we suggest that OvHV-2-linked low abundance of IL-2 transcripts may be a key to further study the pathogenesis of MCF. Indeed, IL-2 plays a critical role in the termination of active immune responses by stimulating the T-regulatory cells (Treg), which are supposed to silence activated, cytotoxic T-cells. In the context of IL-2-deficiency, this function is lacking, which may explain accumulation of activated, dysregulated cytotoxic lymphocytes in various tissues of animals with MCF. Thus, these observations give for the first time a possibility to provide a model for MCF pathogenesis, which can be addressed experimentally.

Finally, our observations are also interesting in the light of co-evolution. The extra sets of Macavirus genes must have been subject to co-evolutionary selective pressure. For example, OvHV-2 circulates only among its reservoir animal species, which remain healthy, but not among species that are susceptible to MCF. Therefore, the virus must have co-evolved to keep its host healthy. Since the extra set of genes is silent throughout MCF, we suggest that they are not factors driving MCF but rather may play a role in protecting their own host species from succumbing to MCF.

MECHANISMS OF BLUETONGUE VIRUS (BTV) EVOLUTION AND THEIR POTENTIAL RELEVANCE TO PHENOTYPIC PROPERTIES OF BTV AND DIAGNOSTIC AND VACCINE STRATEGIES

N. James MACLACHLAN

Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, CA 95616

Bluetongue virus (BTV) is the prototype virus in the genus Orbivirus, family Reoviridae, and the cause of an important insect-transmitted animal disease (bluetongue) that recently has spread throughout much of Europe. BTV has a genome of ten distinct segments of double-stranded RNA, which collectively encode 7 structural (VP 1 – 7), and 4 nonstructural (NS 1, 2, 3/3A) proteins. There are 24 serotypes of BTV currently recognized worldwide, and a likely 25th serotype recently was described in Europe. There is marked sequence diversity among global strains of BTV, and in the viruses that co-circulate in enzootic regions. In contrast, the BTV strains that spread during epizootics are typically genetically similar. Field strains of BTV evolve by both genetic shift and genetic drift, the latter through a complex process of quasispecies evolution and founder effect during the sequential infection of arthropod and mammalian hosts that occurs during virus transmission. Genetic shift occurs through exchange (reassortment) of entire gene segments in animals or insects infected with more than 1 BTV strain or serotype. Over time, certain BTV genes evolve through negative (purifying) selection into region-specific genetic "topotypes".

BTV infection occurs throughout much of the world, often with little associated occurrence of disease. The remarkable genetic diversity of field strains of BTV that occur throughout the world is reflected in differences in their biological properties, including their virulence to animals and perhaps their vector tropism (which determines the global range and distribution of each virus). For instance, viruses of the same serotype can exhibit remarkable differences in their virulence to susceptible breeds of sheep. Similarly, some virus strains such as that the strain of BTV serotype 8 currently circulating in Europe cause disease in multiple animal species, including wild and domestic ruminants, South American camelids, and even carnivores. Although the functional properties and relationships of individual BTV proteins are increasingly defined through research, the genetic basis of the profound difference in the phenotypic properties of individual virus strains is poorly characterized.

Appreciation of the marked genetic differences between BTV strains is also important to the logical design of both vaccine and diagnostic virus detection strategies. The extensive genetic variation amongst field strains of BTV strains means that BTV nucleic acid detection tests, like quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assays, must be designed to detect all BTV strains or they risk giving false negative results with certain viruses. The recent identification of a novel, likely 25th BTV serotype amongst goats in Switzerland only highlights the need for constant vigilance regarding the sensitivity and specificity of virus detection assays to be used in the field and for regulatory purposes pertaining to the international movement and trade of animals.

Vaccines are central to control strategies in regions where virulent strains of BTV are present and cause disease in ruminants. Live attenuated (LA) BTV vaccines have been used for many decades to prevent bluetongue disease amongst sheep in South Africa and the United States, and both inactivated and LA vaccines have been used for protective immunization of cattle, sheep and other animal species in Europe. These vaccines confer serotype-specific immunity in immunized ruminants, however, LA vaccine viruses also can be acquired by insect vectors and circulate in nature (or contribute gene segments through reassortment). Furthermore, some LA BTV vaccines readily cross the placental to cause reproductive losses and developmental defects, which is especially disconcerting if these viruses can be acquired and transmitted by insect vectors. Lastly, current virus detection and serological tests do not distinguish infection of ruminants with LA vaccine or true field viruses; indeed, LA vaccine viruses that are acquired by insect vectors can very likely become "field" viruses. Inactivated vaccines, including recombinant BTV vaccines that include the BTV outer capsid proteins, potentially avoid many of the problems inherent to LA vaccines.

RECENT TRENDS IN THE DIAGNOSIS OF EMERGING AND RE-EMERGING TRANSBOUNDARY ANIMAL DISEASES AND IN THE DETECTION OF FOOD- AND WATERBORNE INFECTIONS

Sándor BELAK

Joint Research and Development Division, Departments of Virology, Swedish University of Agricultural Sciences (SLU) and National Veterinary Institute (SVA), The Collaborating Centre for Biotechnology-based Diagnosis of Infectious Diseases in Veterinary Medicine of the OIE (World Organisation for Animal Health), Ulls väg 2B, SE-751 89 Uppsala, Sweden

Transboundary animal diseases (TADs), such as foot-and-mouth disease, classical swine fever, African swine fever, African horse sickness, bluetongue, Newcastle disease and avian influenza are highly pathogenic infectious maladies that migrate across boundaries between various geographic regions or countries, causing very high economic and socioeconomic losses worldwide. Due to the highly infectious character of these pathogens, globalisation, strongly intensified and accelerated animal and human traffic, climatic changes and other factors, the TADs show a very rapid global spread today. These diseases are fought at the international level by international organisations and bodies such as the World Organisation for Animal Health (OIE), the European Commission, the FAO and the IAEA, among others. OIE Collaborating Centres are centres of expertise in a specific designated sphere of competence, with a mandate to provide their expertise internationally. In this presentation the recent activities and achievements of the OIE Collaborating Centre for Biotechnology-based Diagnosis of Infectious Diseases in Veterinary Medicine, at the National Veterinary Institute (SVA) and at the Swedish University of Agricultural Sciences (SLU), together with selected achievements of our collaborative partner laboratories, are briefly summarised. Recent trends for detection of pathogens in food- feed- and waterborne diseases are also discussed. The novel technologies, developed and/or adapted and used at our OIE Collaborating Centre and at our partner laboratories include a) powerful methods of sample preparation and enrichment; b) comparison of the capacities and the performances of various nucleic acid extraction and pipetting robots; c) comparison of the performance and stability of various real-time PCR machines; d) development of robust and high throughput real-time PCR assays, such as Primer-Probe Energy Transfer System, which, in comparison to TaqMan is more properly tolerating nucleotide mismatches in the targeted nucleic acids, thus, allowing a robust and general diagnostic system; e) development of further novel real-time PCR assays, such as the light upon extension fluorogenic primer (LUX RT-PCR) system, which provides high specificity, sensitivity and relative simplicity as a novel, rapid and cost-effective diagnostic tool for TAD surveillance and monitoring programs; f) development of the LATE PCR assays, which facilitate the use of portable PCR machines and the front-line diagnosis of TADs; g) elaboration of isothermal amplification methods, such as Invader and LAMP assays, which are run on simple and moderate-price thermoblocks, without the need for expensive PCR machines; h) development of multiplex and multi PCR platforms, which allow the simultaneous detection of several pathogens in a single clinical sample, or disease scenario, allowing a very rapid complex diagnosis; i) solid and liquid phase microarrays, including the use of padlock probes and the Luminex systems; which further increase the complexity of the diagnosis, by allowing to test as many as 100-500 pathogens/pathogen variants or other biological factors, such as antibiotic resistance genes, cytokine expression profiles simultaneously in a single complex platform; j) full-genome sequencing methods for the improved investigation and characterisation of viral populations, determination of complete genome structures, including pathogenicity markers and other biological markers; n) powerful methods of phylogeny, such as the Bayesian approach, which allows the confident placement of new strains in the current classification of viruses into genotypes and subgenotypes; m) methods to detect emerging and "unknown" viruses, as novel tools to investigate emerging new pathogens and outbreak scenarios; n) viral metagenomics, when viral particles from uncultured environmental and clinical samples are purified and their nucleic acids are randomly amplified prior to subcloning and sequencing, in order to detect known and unknown viruses and to investigate the composition of the animal virome; o) proximity ligation assays as novel tools for the sensitive investigation of viral antigens by using monoclonal antibodies coupled to oligonucleotides and amplifying these structures by PCR, providing high sensitivity and specificity for viral antigen detection and identification; p) dip-stick tests, lateral flow devices for "on site" diagnosis of TADs in the front line of the outbreaks; r) improved assays for antibody detection, including Luminex antibody detection systems, to estimate the antibody responses in single and in multiple forms of infections.

By using the above listed methods in individual or in complex assays, a wide range of novel diagnostic approaches were developed at the OIE CC and the partners laboratories, for the improved detection of a wide range of TADs, see for example the LAB-ON-SITE project of an EU-based large consortium: www.labonsite.com. The main principles and the majority of the results are summarised in this keynote lecture presentation and discussed during the 2009 congress of the ESVV with the members of the Society.

These are examples, which illustrate the expanding arsenal of diagnostic methods used for the direct and for the indirect detection and identification of various viruses and other pathogens. The listed methods, in various variations and complexity, are applied today in our and in partner laboratories for the improved diagnosis of TADs and endemic viral diseases, for DIVA approaches (differentiate infected from vaccinated animals), to detect virological and other microbiological contaminations in biological products. For example, pestiviruses are frequently contaminating foetal calf serum (FCS) batches. The majority of the contaminating viruses belong to bovine viral diarrhoea virus (BVDV) types 1 or 2. In addition, our recent studies revealed and confirmed the presence of atypical bovine pestiviruses (suggested term is BVDV-3) in foetal calf serum bathes, indicating that these emerging, or so far undetected, pestiviruses have to be seriously considered in the bioindustry. It has to be strongly emphasized that sensitive detection and identification methods are highly needed in order to guarantee the virus-free status and biosafety of biological products, which contain FCS, such as vaccines, cell culture systems, diagnostic systems, practically all bioproducts, which are based on the use of FCS.

In addition to diagnosis of TADs and endemic viral diseases, some of these methods are used for the detection and identification of emerging pathogens in food and in feed products. In order to improve food safety, PriProET and novel TaqMan real-time PCR assays were developed at our laboratories for the improved detection of hepatitis E virus (HEV), which has not only food safety, but also zoonotic importance.

In order to study the genomic structure of HEV as well as the relationship of different variants of this virus, the full-length genomic sequence of a HEV of European porcine origin, termed swX07-E1, was determined and it was found that this virus, originating from Europe, belongs to genotype-3 HEV. Virus swX07-E1 clusters with variants from Japan, Mongolia and Kyrgyzstan in subgroup 3c, but it is divergent from the prototype US HEV. This phylogeny, based on full-genome sequencing, indicates that swX07-E1 represents a new subgroup of genotype-3. The study proves that analysis of full-length sequences is a useful to discover new subgroups of viruses, as it was shown in this model for HEV.

In summary, the novel laboratory-based and the "on site" detection systems, being introduced at our OIE Collaborating Centre and at our partner laboratories, provide powerful novel means to combat the TADs and to improve food and feed safety, by following the "One World One Health" principle at a global scale.

CIRCOVIRUSES – A COMPARATIVE APPROACH

Daniel TODD

Agri-food and Biosciences Institute, Stormont, Belfast BT4 3SD, U.K.

Circoviruses comprise small, non-enveloped, spherical viruses that are unique among animal viruses in having circular, single-stranded DNA genomes, the smallest possessed by animal DNA viruses. The diseases caused by the circoviruses porcine circovirus type 2 (PCV2) and chicken anaemia virus (CAV) are of considerable economic importance. As a consequence most is known about these 2 circoviruses, while comparatively little is known about the remaining 11 species. The purpose of this presentation is to provide an update on research findings on a variety of aspects ranging from toxonomy to disease, that I think are relevant to the study of circoviruses as a whole. A comparative approach will be adopted with focus being put on how knowledge gained from studying all circoviruses may be of use to those tasked with investigating particular and future circovirus examples.

The family *Circoviridae* comprises 2 genera. CAV is the only member of the genus *Gyrovirus*, while the genus *Circovirus* presently comprises viruses from pigs (porcine circovirus types 1 and 2; PCV1, PCV2), psittacines (beak and feather disease virus; BFDV), pigeons (PiCV), geese (GoCV), canary (CaCV) and duck (DuCV). Additional viruses of this genus have been recognised in finch, gull, starlings, ravens and swans. Of the 13 circovirus species identified to date, 11 are associated with avian hosts, while the pig remains the only mammalian host. CAV particles (~25nm) are larger than those of other circoviruses (~20nm) and display a more pronounced surface structure. Importantly from a taxonomic point of view, the genome organisation of the CAV genome (2.3kb) differs substantially from those (1.7-2.0kb) of other circoviruses. CAV has a negative sense, single-stranded genome from which a major 2.0kb transcript is produced. This transcript contains 3 ORFs each of which is translated, producing VP1, the 52kDa capsid protein, VP2, a 28kDa non-structural viral phosphatase and VP3, a 14kDa non-structural protein, named "apoptin" on the basis of its ability to cause apoptosis. CAV shares has genome organisational features with torque teno viruses (TTV) and TTV-related viruses, which are separately classified in the floating genus *Anellovirus*, quite distinct from the family *Circoviridae*. However, these similarities have prompted speculation that CAV, the only gyrovirus, may be transferred from the family *Circoviridae* to a yet to be established, new virus family that will also include the anelloviruses.

Our understanding of the molecular biology of viruses belonging to the genus *Circovirus* is based on research performed on PCV1 and PCV2, which remain the only genus members that can be propagated in cell culture. These circoviruses have ambisense genome organisations in which major and minor transcripts are generated from each of the 2 strands comprising the double-stranded RF. ORF C1 (ORF 2), located on the complementary-sense strand, encodes the capsid protein while differentially spliced transcripts of ORF V1 (ORF 1), located on the virus-sense strand, encodes 2 forms of the replication-associated protein (Rep, and Rep`). In the case of PCV2, a minor ORF located on the complementary strand has been reported to encode a small protein with the ability to cause apoptosis. The genomes of the remaining avian circoviruses are similarly organised with regards to the locations of the 2 major ORFs, but it remains unknown whether other minor ORFs possessed by some of these are produced as biologically active proteins. One of the most distinctive features of the circovirus genomes is the 5` intergenic region, which is located between the start codons of the C1 and V1 ORFs, and which contains the origin of replication. A distinctive stem-loop structure, containing the conserved nonamer sequence, where cleavage occurs to initiate rolling circle replication, is located within the origin together with repeat sequences which are found adjacent to the stem-loop and which constitute the binding sites for the Rep and Rep` proteins.

Based on the suggestions of the International Committee on Taxonomy of Viruses (ICTV), a virus is considered to be a new member of the genus *Circovirus* if its complete genome nucleotide similarity is less than 75% and its capsid protein sequence similarity is less than 70% when compared to any known circovirus species. Genome comparisons have shown that the virus species comprising this genus vary with regards to the lengths of their genomes, their 5` and 3` intergenic regions and the sizes of their Rep and Capsid proteins. Pairwise comparisons of the circovirus species have shown that Rep proteins, which contain conserved amino acid motifs that are associated with rolling circle replication, share higher levels (38.2-86.0%) of amino acid identity than the capsid proteins (17.1-65.5%). Higher intra-species genome nucleotide sequence diversities were observed with avian circoviruses such as BFDV (infecting psittacine birds) and PiCV than with the PCVs. Based on genomic sequences, PCV2s can be subdivided into two major groups, designated Group 1 and Group 2, each containing several clusters, but none of these was apparently associated with disease status or geographic area. Examination of the capsid protein sequences of other circoviruses, such as PiCV, have identified variable regions that may represent sites of antigenic variability. A phylogenetic analysis revealed a strict co-evolution of circoviruses with their hosts, and this is consistent with the view that circovirus species have narrow host ranges.

The clinico-pathological features of CAV-assocated disease differ from those that are associated with the other circoviruses. Although PCV1 is non pathogenic, PCV2 is causally associated with postweaning multisystemic wasting syndrome (PMWS), now renamed porcine circovirus disease (PCVD), an economically important disease of 8-15 week-old piglets characterised by clinical signs of wasting, reduced weight gain, jaundice, respiratory complications and lymphoid depletion. The diseases associated with the avian circoviruses including BFDV, PiCV and GoCV have clinico-pathological features in common with PCVD. These include ill-thrift, reduced weight gain and an increased susceptibility to secondary pathogens that is indicative of underlying immmunosuppression. Common histopathological features include lymphoid depletion, infiltration of histiocytes and the detection of intracytoplasmic inclusions within macrophages. Most avian circoviruses infections were originally diagnosed by histology or electron microscopy. However, due to their lack of growth in cell culture and the absence virus-specific antibodies for use in antigen detection methods, subsequent diagnostic methods have included *in situ* hybridisation, dot blot hybridisation and PCR. Applications of these DNA-detecting methods have indicated that circovirus infections are highly prevalent and widespread in their respective avian hosts. Although many infections appear to be subclinical, the DNAs of avian circoviruses such as PiCV and GoCV have been detected in some birds at very high levels (>10^{12} genome copies/ g tissue) and in a wide range of tissues, often in the absence of histological changes. By analogy to PCV-2 infections, it seems probable that, in such cases, the circoviruses are of importance in the disease manifestations including growth retardation and increased susceptibility to secondary pathogens.

There is a strong likelihood that circoviruses will be recognised in additional avian species, including commercially produced poultry and game birds. If this proves to be the case, it is possible that they will cause variable degrees of immunosuppression, which in turn will adversely affect commercial performance. Their involvement in subclinical and multifactorial diseases, in which clinical outcome can depend on the secondary pathogens present, may make circoviruses typical of a new generation of viruses with which the veterinary virologist will be challenged.

AFRICAN SWINE FEVER. A DANGEROUS RE-EMERGING DISEASE

José-Manuel SÁNCHEZ-VIZCAÍNO

OIE ASF Reference Laboratory, Universidad Complutense of Madrid (UCM), Spain

African Swine Fever (ASF) is one of the most important infectious diseases of the pig that in the last years has undergoing an expansion in its distribution. ASF it is a viral swine disease caused by an icosahedral complex DNA virus that affects only the porcine species of all breeds and ages. The disease was described for the first time in Kenya by Montgomery in 1921 when the virus spread from infected warthogs (*Phacochoerus aethiopicus*) to domestic pigs (*Sus scrofa*) causing a disease with 100% mortality. Since then, ASF has been reported in many countries in Africa, namely: Angola, South Africa, Sudan, Mozambique, Zimbabwe, São Tomé and Principe, where ASF has been shown to be endemic. In 1957, ASF was detected for the first time outside the African continent when a peracute form with a mortality rate of almost 100% occurred in Lisbon. In 1960, the disease reappeared near Lisbon, apparently as a new outbreak, and spread through the rest of Portugal, reaching Spain the same year. The disease in Portugal and Spain was endemic until 1995 when, after the implementation of a very stringent eradication programme, both countries were declared ASF-free. Recently ASF virus was spread in several new African and East European countries affecting domestic and wild swine.

ASF virus is maintained in Africa by a cycle of infection between wild boars and soft ticks. In some of these wild boars the ASF virus infection is characterized by low levels of virus in the tissues and low or undetectable levels of viremia but it is enough for ticks transmission to domestic pigs. This disease cycle makes it very difficult to eradicate ASF in Africa. In Sardinia, (Italy) where ASF is still present, no inapparent infection in wild or domestic pigs and no soft tick populations have ever been observed. European wild pigs are as susceptible as domestic pigs and their role in epidemiology is similar to domestic infected pigs. However, in Sardinia, recovered carrier ASF infected domestic pigs can be detected on endemic countries and its epidemiological role is very important for ASF eradication. The serological control of these animals has been very successful in the eradication program of Spain. Due to the lack of neutralizing antibodies in ASF, the correlation between a new outbreak and the possible source of virus strain has been very difficult to establish. Even the use of the hemadsorption inhibition reaction is not conclusive. The molecular epidemiology, comparing the patterns of DNA from different ASF isolates, appear most promising, especially for the differentiation between African and European ASF strains. However, it is epidemiologically well established that the entrance of ASF virus in a free country is related to the entrance of uncooked infected pork through international ports or airports where garbage containing uncooked pork can be found and used for pig feeding. Once ASF is established in domestic swine, infected carrier-animals are the most important source of virus dissemination for susceptible pigs. At present, no treatment or effective vaccine against ASF virus is available.

In this presentation a review of the actual epidemiological situation of ASF virus is review. The probability of spread to other European countries is evaluated as well as the epidemiological situation of ASF an endemic countries in particular the different scenarios observed in east and west Africa. The analysis of the notified ASF-outbreaks from January 1998 to March 2009 confirms the expansion of the virus to eight new countries and a significant (70% outbreaks notifies in 2007 and 2008) increase of the number of outbreaks in last two years. The increase of the incidence in the African countries, and its expansion to new areas (especially the Caucasian region) suppose a serious risk for the potential introduction of the disease in the European Union.

An epidemiological analysis of the disease evolution, that contributes to understand the epidemiology of the disease and help in the application of preventive and control measures not only in endemic areas, but also in the free ones.

EU SUPPORTED RESEARCH IN ANIMAL HEALTH: PAST, PRESENT AND FUTURE

Isabel MINGUEZ-TUDELA

Directorate for Biotechnologies, Agriculture, Food Research, Directorate General for Research, European Commission

Keywords: EU Agricultural research, Framework Programmes, European Research Area

Introduction

The EU supported research in animal health and in particular in major infectious diseases has to be considered in a double context. Firstly in the context of the development of the animal health legislation within the Common Agricultural Policy (CAP) and later within the Community Animal Health Policy (CAHP). Secondly in the context of the European research policy before and after the establishment of the Framework Programmes (FP) for Research, Technological Development and Demonstration activities as well as of the concept of the European Research Area (ERA).

With regard to the CAP, the legal basis was already established in Article 41 of the 1957 EEC (1) Treaty which foresaw provisions for research intended to boost agricultural productivity. In 1974, the Council Resolution on coordination of agricultural research established the Standing Committee on Agricultural Research (SCAR).

EU research policy

With regard to the Community's research policy, the foundation lies in the Founding Treaties: 1951 ECSC (2) Treaty, the 1957 Euratom Treaty (3) and the 1957 EEC Treaty (1). In the latter, in addition to Article 41 mentioned above for agricultural research, Article 235 provided a general legal basis for action in a variety of sectors, including research and technology for which no specific constitutional provision was originally made. This article allowed the launching of several research programmes in the 60s and 70s in areas considered as priorities. In 1983 the Council (4) approved the principle of FPs in order to organise under a single "framework" all the research activities with a budget covering several years. The FPs defined the scientific and technical objectives for transnational collaborations and the selection criteria for the corresponding multiannual period. In the selection process, special attention was given to activities which could contribute to the definition or implementation of Community policies. Later, The Single European Act of 1987 included a specific chapter on research policy in the EEC Treaty.

The 1st FP 1984-1987 was followed by successive FPs of 4 years except the current 7th FP which has a 7 year duration 2007-2013. The main evolution of the FPs relate to the increasing budget, the extension of the scientific and technical fields and the diversification of support mechanisms and methods of implementation.

In 2000 the concept of a European Research Area (ERA) became an overall political objective and the reference framework for research policies to overcome fragmentation of the research activities across Europe. Realising ERA is an integral part of the Lisbon Strategy for Growth and Jobs and a component of the EU´s response to the challenges posed by globalisation. The concept of ERA combines 3 components: 1) the creation of a European internal market for research where researchers, knowledge and technology would circulate freely (Fifth freedom after goods, people, services and capital); 2) an area of coordination of national and regional activities, programmes and policies and 3) an area for the implementation and funding of Europe-wide initiatives.

ERA policy actions and the FPs programmes and activities reinforce each other. The 7th FP, considered the largest and most ambitious multinational research programme in the world underpins the ERA by deploying a range of funding schemes through the Cooperation, Ideas, People and Capacities specific programmes. The 7th introduces in particular two new major developments: the European Research Council (ERC) and the Joint Technology Initiatives (JTI). The ERC funds fundamental research projects proposed by individual teams in fast emerging and highly interdisciplinary fields and carried out without the obligation of transnational partnerships (Ideas programme). The JTI are public private partnerships where research, industry and other stakeholders combine their energies to solve major technological and societal problems. Five JTIs have developed from the European Technology Platforms (ETPs).

The Green Paper "The ERA: New Perspectives" of April 2007 gave an impetus to the ERA project through its public consultation. An overall governance process of ERA -the Ljubljana process (April/May 2008)- is currently being set up based on a shared "2020 ERA Vision" agreed by the Commission and Member States in December 2008. Five specific initiatives to support ERA were launched in 2008 addressing: 1) researchers´careers and mobility; 2) research infrastructures; 3) knowledge sharing; 4) joint programming (building on the ERA-NET scheme and initiatives based on Article 169) and 5) international science and technology cooperation.

EU Animal health research

Before the FPs, EU research in infectious diseases of animals was included within the agricultural research. The first Community programme on agricultural research goes back to the AGRIRES 0C programme (1973–1976) to "*coordinate efficiently Community research into the virology of classical and African swine fevers in order to maintain and increase productivity in the swine breeding sector*". Laboratories and research centres of Member States DE (GFR), BE, FR, IT, NL,UK,IE and DK and two associated laboratories from ES and PO. It was followed by AGRIRES 1C (1976-1978), AGRIRES 2C (1979-1983) and AGRIRES 3C (1984-1988) all three covering agricultural research in which activities related to viral diseases of livestock (e.g. avian and bovine leucoses) were foreseen. These programmes included both coordination of research and implementation of joint activities and were aimed to help the improvement of productivity and the removal of obstacles to a more efficient operation of the CAP (in this case the creation of the common market for animals and animal products).

In the FPs, research in infectious diseases was also undertaken in the specific programmes for agriculture research CAMAR in 2nd FP, AIR and FAR in 3rd FP, FAIR in the 4th FP, Priority 5 and "Support to Policies" in the 6th FP and Theme 2 of the current 7th FP as well as in the various international S&T cooperation programmes (INCO) from 1983 to 2006. In the 5th FP however, it was included in the same area as human infectious diseases -Key Action 2 of the Quality of Life Programme.

The majority of EU supported projects aimed to improve or develop new tools and knowledge for the control and prevention of animal diseases incorporating the latest scientific developments in order to provide scientific basis for the development of the animal health legislation necessary for the single market and the free circulation of animal and animal products. Most of the projects addressed major diseases which were subject to Community legislation, the majority included in the former list A of OIE and others of serious economic concern for farmers (e.g. FMD, CSF, ASF, BT, AHS, HPAI, SI, BVDV, PRRS, PCV etc.). European research consortia have been built over the years with support in successive FPs. This support has allowed the breaking of barriers and the building of bridges between European research groups of different cultural backgrounds. The research consortia are progressively including an increasing international dimension, a closer collaboration with the animal health industry and international organisations (OIE, FAO and WHO) and groups working in human health ("One health"). Research, both EU and national, in animal health is the 4th pillar "Science, Innovation and Research" of the Action Plan for Animan Health Strategy " *Prevention is better than cure*". Two important initiatives need to be highlighted: the European Technology Platform on Global Animal Health and the ERA-NET EMIDA for the coordination of national programmes on research in animal health. The latter builds on the work done in the SCAR. These two initiatives are in line with the latest developments of the ERA and will contribute to setting the basis for the new research approach envisaged in the 2020 ERA vision with regard to animal health research.

Ackowledgements

Michel André and Jeremy Bray, EC RTD.

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NOTHING IN VIROLOGY MAKES SENSE EXCEPT IN THE LIGHT OF EVOLUTION

Marian C. HORZINEK

Bilthoven/Netherlands

The title is a homage to Darwin, in this year specially dedicated to his ground-breaking insights; it is also a paraphrase of Theodosius Dobzhanski's famous quote (change 'virology' to 'biology'). In this presentation, evolution will be viewed at various levels: considering the virus in an infected organism, its genetic changes during epidemics in a population, and - in a more philosophical approach - as the evolution of virologists when they leave the bench and become writers and directors of research. Finally, the evolution of our discipline will be considered - from its birth after identification of the *contagium vivum fluidum* as a novel form of infectious agent to its present-day importance as a toolbox for molecular geneticists, cell biologists, immunologists et al.

The virological examples in this presentation are taken from the Coronaviridae, a family of viruses with the largest RNA genome known to science and consequentially high mutation rates. In an infected organism, the example of feline infectious peritonitis has shown how genomic changes make a relatively harmless enteric pathogen into a killer. This is a non-contagious infectious disease, because the killer mutants arise in the individual cat and are normally confined to its organism and not transmitted. - In a population, the SARS example is notorious for proving that an expansion of the coronaviral host spectrum, in this case from palm civets to man, can escalate to become a public health concern; the wild fauna as an inexhaustible reservoir of 'new' viruses is considered, in particular the role of bats. Of the more than 4,600 recognized species of mammals, about 24% are bats. A recent pilot study of coronaviruses in bats captured in Colorado revealed three new coronaviruses; members of 14 virus families have been isolated from bats (85 viruses), and this order of vertebrates definitely deserves more veterinary and public health attention.
Also virologists evolve in their individual careers, starting as specialists with in-depth knowledge focused on one or a few phenomena, eventually becoming generalists, with a transition from experimental to review publications and keynote lectures - like this one. The two are faces of the same medal, and both are essential to the continuity and prestige of the discipline. Of particular concern is the loss of traditional techniques from the experimental arsenal - to be replaced by more 'sexy' molecular means. While these incontestably have contributed to the quantum leap in virological insight during the last decades, simple, straightforward methods - from animal infection experiments to neutralization tests - must not be forgotten. Progress in methodology has always preceded scientific insight, and these techniques should not disappear from the teaching curriculum of veterinary and graduate schools, if only to show the historical evolution of the discipline. - On the other hand, generalists are essential in translating bench data into concepts and principles to be communicated in review articles to the peers from other walks of virology as well as to science journalists in a format intelligible for the lay person. Education of the public in the concepts of infectious diseases, their prevention, epidemic spread and dynamics are of societal importance, as the present influenza epidemic has emphasized. Virology needs generalists as much as it needs specialists; as the pun summarizes: the 'laboratory' spawns bench workers ('labor') and speakers ('oratory').

When looking at the achievements of veterinary virologists for the entire field of natural sciences since 1898, our science has a prestigious pedigree indeed. From the discovery of filterability of foot-and-mouth virus to the definition of cellular immunity by a veterinary Nobel laureate, the role virology played for biological sciences has been paramount. The European Society of Veterinary Virology offers its members a platform with a wide range of expertise, as the present congress program shows.

MICROBE HUNTING IN THE 21ST CENTURY

W. Ian LIPKIN

Differential diagnosis of infectious diseases is becoming increasingly important in clinical medicine and public health. Factors in raising global concern with respect to acute infectious diseases include burgeoning international travel and trade, political instability and bioterrorism, climate change and its effects on vector distribution, and the emergence and reemergence of zoonoses. The ability of agents rapidly to expand their geographic range and appear in unexpected locations is well illustrated by the global spread of the human immunodeficiency virus, the transfer of West Nile virus to the western hemisphere and its subsequent dissemination throughout North and South America, the recent emergence of chikungunya virus in Europe, and of multidrug resistant tuberculosis worldwide. Unexpected emergences have also occurred in the context of organ transplantation and immunosuppression. There is also a growing appreciation for a potential role for microbes as primary or co-factors in chronic cardiovascular, endocrine, neurodevelopmental and neoplastic disorders. Whereas the absence of effective therapies once made the diagnosis of viral infection primarily an academic exercise, the expanding armamentarium of countermeasures tailored to specific viruses, including small molecules, RNAi, therapeutic antibodies and vaccines, affords new opportunities to significantly reduce morbidity, mortality and health care costs due to viral infections.

Although culture and serology remain vital in diagnostic clinical microbiology and pathogen discovery, sequence-based methods have clear advantages with respect to speed, cost and portability. Furthermore, many are easier to implement because they require less investment in infrastructure and training than culture techniques. Lastly, sequence-based methods may succeed in instances where fastidious requirements confound cultivation.

Active collaboration between clinicians and laboratorians will be key to success in this new era. The most advanced technology will fail if samples are collected without attention to nucleic acid and protein lability. Data will be uninterpretable without accurate information on clinical course and sample provenance. In chronic diseases, wherein complex mechanisms such as early exposure and/or genetic susceptibility may contribute to pathogenesis, the most substantive advances in linking microbes to disease are likely to come from investments in prospective serial sample collections and an appreciation that many conditions reflect unfortunate host–microbe intersections.

In this lecture I will discuss mechanisms of microbial pathogenesis, routes to proving causation beyond Koch's postulates, and a staged strategy for microbial surveillance and discovery. In reviewing the strengths and limitations of various analytical platforms, I will provide examples that illustrate how each platform can be used to investigate clinical problems. I will also describe models of gene-environment-timing interactions and their implications for medicine and public health.

NOVEL "SWINE-ORIGIN" H1N1 INFLUENZA IN PEOPLE: THOUGHTS FROM A SWINE FLU VIROLOGIST

Kristien VAN REETH

Laboratory of Virology, Faculty of Veterinary Medicine, Gent University, Salisburylaan 133, 9820 Merelbeke, Belgium

A novel H1N1 influenza virus emerged in humans in Mexico and North America in March and April 2009 and subsequently spread to Europe and other continents. Phylogenetic analyses showed that the virus is a reassortant of at least two existing swine influenza viruses (SIVs) (Garten et al. 2009 [3], Smith et al. 2009 [9]). Six gene segments were similar to those of triple reassortant SIVs circulating in pigs in North America. The genes encoding the neuraminidase and matrix proteins, on the other hand, were most closely related to those in SIVs circulating in Europe or Asia. Influenza viruses of H1N1, H3N2 and H1N2 subtypes are enzootic in swine populations worldwide, but the antigenic and genetic constellation of the predominant viruses is entirely different in North America versus Europe or Asia (Olsen et al. 2006 [7]). In North America, viruses of the "classical" H1N1 lineage were the dominant cause of swine influenza until the late 1990s and H3N2 viruses have only become widespread since 1998. The predominant H3N2 viruses were so-called triple reassortants with genes of classical swine, avian and human origin. These viruses further reassorted with classical swine H1N1 viruses, leading to H1N2 and reassortant H1N1 viruses (Vincent et al. 2008 [11]). In Europe, the predominant H1N1 SIVs have an entirely avian genome and were introduced from wild ducks to pigs in 1979 (Pensaert et al. 1981[8]). These avian-like H1N1 viruses have established a stable lineage and have replaced the classical H1N1 viruses soon after their introduction. They are currently cocirculating with H3N2 and H1N2 SIVs, which also differ from their counterparts in the US. The European swine H3N2 viruses have been derived from the human virus causing the "Hong Kong flu" pandemic in 1968, but their internal genes have been obtained through reassortment with the avian-like H1N1 virus. The dominant H1N2 viruses retained the genotype of these reassortant H3N2 viruses, but they have acquired an H1 gene through reassortment with a human H1N1 virus from the early 1980s (Brown et al. 1998 [2]).

While the well-known SIVs cause only sporadic human infections (Myers et al. 2007 [6]), the novel H1N1 virus transmits efficiently between humans. At this time of writing 94512 cases and 429 deaths have been reported in a total of over 70 countries, and the World Health Organization declared the first flu pandemic in 41 years. The novel H1N1 virus most likely emerged in pigs, though the specific virus has not yet been reported in swine populations anywhere in the world. In the European swine population, the virus appears to be absent, as it has never been reported by the European Surveillance Network for Influenza in Pigs (ESNIP). Some fear that this novel virus will become introduced in swine populations through infected humans, especially if their numbers continue to increase. Recent experimental infection studies have confirmed the susceptibility of pigs to the virus and its capacity to transmit between pigs (Brookes et al. 2009 [1], Lange et al. 2009 [5]). One crucial question, however, is to what extent pre-existing immunity against SIVs that are enzootic in Europe may protect pigs against the novel H1N1 virus. Interestingly, preliminary investigations of sera from pigs immune through infection or vaccination with European SIVs revealed greater cross-reactivity than one would expect based on antigenic and genetic analyses as such. In the first part of my lecture, I will focus on the origin of the novel H1N1 virus and present my personal viewpoint on its significance for the swine industry as well as for human health.

Pigs have been assigned a role in the generation of pandemic influenza viruses for humans for decades. In theory, such pandemic viruses could emerge following modification of an established swine strain, adaptation of a strain of avian origin to mammals, or reassortment between avian and human influenza viruses (Ito et al. 1998 [4]). It is a classical theory that pigs are more susceptible to avian influenza viruses than humans, and that they are essential intermediary hosts for the introduction of avian viruses or avian virus genes in the human population. The recent outbreaks of H5N1 avian influenza in Asia, as well as the current novel H1N1 pandemic have started to change our classical viewpoint of the role of the pig in the transmission of influenza viruses to humans. H5N1 and other wholly avian viruses have been able to infect pigs under experimental conditions and in nature, but there is also a strong barrier to infection of pigs with such viruses (Van Reeth 2007 [10]). In addition, avian influenza viruses largely lack the capacity to spread between pigs. The human cases of infection with H5N1 were invariably due to direct contact with infected poultry, and the virus still fails to spread efficiently between humans. On the contrary, swine-adapted influenza viruses of various subtypes and genotypes have been shown to transmit to humans on several occasions, but second generation transmission was extremely rare (Myers et al. 2007 [6]). The novel H1N1 virus is the first virus of presumed swine origin that resulted in efficient transmission between humans. Unfortunately, we still have a very limited understanding of what is needed for efficient replication in and adaptation of avian influenza viruses to pigs. Similarly, it remains unknown what factors trigger transmission of influenza viruses from pigs to humans at the physiological and molecular level, or what is needed for the further transmission of such viruses between humans. In the second part of my lecture, I will review our current knowledge about these issues.

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Oral presentations

TOGGENBURG ORBIVIRUS: GENETIC CHARACTERIZATION, DIAGNOSTICS AND EPIDEMIOLOGY OF A NOVEL BLUETONGUE VIRUS DETECTED IN GOATS FROM SWITZERLAND

Martin HOFMANN, Valérie CHAIGNAT, Gabriella WORWA, Barbara THUER

Institute of Virology and Immunoprophylaxis, Mittelhaeusern, Switzerland

Keywords: orbivirus, phylogeny, prevalence serotype, real-time RT-PCR

Introduction and objectives

A novel orbivirus termed "Toggenburg Orbivirus" (TOV) has recently been detected by real-time RT-PCR (rRT-PCR) in clinically healthy goats in Switzerland (1, 2). In vivo studies showed that TOV does not cause clinical and pathological signs in exerimentally infected goats and only mild pathological lesions in sheep (1). Sequence analysis of 7 of the 10 genome segments suggests that TOV might represent a 25th serotype of bluetongue virus (BTV) (2). Here, we present results of the nucleotide sequence analysis of the coding region of all 10 viral RNA segments. Based on the VP2 sequence a TOV-specific rRT-PCR was developed. A synthetic positive control was produced due to the inability to propagate TOV in cell culture. Preliminary results from an epidemiological survey are shown as well.

Materials and methods

BTV rRT-PCR: For screening purposes a BTV RNA segment 10-specific rRT-PCR (3) was used, whereas for confirmatory purposes 4 different rRT-PCRs specific for segments 1 (4, 5) or 5 (5) were applied.

Sequencing: Each of the viral genome segments was amplified by RT-PCR using primers binding to the conserved ends of BTV RNA segments. PCR products were cloned into a plasmid vector and sequenced by external and insert-specific internal primers.

TOV-specific rRT-PCR: Based on the sequence analysis of the VP2 of several TOV isolates, primers and a probe were designed. This TOV-specific rRT-PCR was used as a confirmatory test for goat samples scoring positive in the BTV screening test (3). Furthermore, the entire VP2 gene was cloned and used to make a synthetic double-stranded RNA positive control by transcribing RNA in both directions.

Prevalence study: Serum samples collected for routine diagnostics purposes or in the context of a caprine arthritis encephalitis surveillance study from numerous goat herds throughout Switzerland, mainly from the southern cantons of Ticino, Graubünden and Valais were tested for the presense of TOV antibodies by BTV-specific ELISA (previously shown to cross-react with TOV antibodies). Seropositive samples were then tested by rRT-PCR for TOV-specific RNA.

Results

All TOV-positive samples were initially detected by the pan BTV rRT-PCR targeting segment 10 of the viral genome (3). None of the other commonly used rRT-PCRs (4, 5) yielded a positive result.

When all 10 viral genome segments were sequenced and sequences were aligned with the respective genes of other orbiviruses, phylogenetic analysis reveiled that TOV is more closely related to BTV than to any other orbivirus. However, TOV could not be assigned to any of the known 24 serotypes of BTV. Interestingly, all genes coding for structural proteins (VP2, VP5, VP6, VP7) were placed inside the BTV serogroup in the dendrogram, whereas the remaining genes were more distantly related to BTV.

In order to develop a TOV-specific rRT-PCR that is able to differentiate TOV from BTV, and to obtain data on the genetic variability of the VP2, a 281 nucleotide (nt) fragment of segment 2 from all 12 TOV isolates found so far was cloned and sequenced. Despite a certain extent of sequence variability among the TOV isolates, they were found to be highly similar (267/281 nt identical), but distinct from any known BTV sequence. Based on these sequence data, rRT-PCR primers and a probe that bind to conserved regions within the sequenced TOV S2 fragment were subsequently designed. A synthetic double-strand (ds) RNA positive control for this rRT-PCR was produced by cloning the entire VP2 ORF into the pCR4-TOPO vector, followed by in vitro transcription of both RNA strands from either end, and hybridizing these RNAs to each other. dsRNA diluted 10e-8, which yielded an average Ct value 27, was finally used as positive control.

Since TOV was originally found in two unrelated goat flocks in Switzerland, approximately 2000 sera collected early in 2008 from BTV-unvaccinated, healthy goats were tested for the presence of BTV antibodies and viral RNA. Whereas only a few individual samples were positive north of the Alps in Switzerland, a seroprevalence of 72% was found in the canton Ticino, and of 18% in the canton Graubünden. TOV rRT-PCR positive samples were identified repeatedly in these cantons as well. Four more TOV-positive samples (antibody as well as viral RNA) were found in goats originating from northern Italy and southern Germany. Additional TOV-positive samples have also been identified in northern Italy by IZS Brescia (Antonio Lavazza, personal communication).

Discussion and conclusions

We have recently detected a novel orbivirus termed TOV in healthy goats (1, 2), which, based on its genetic organization, is closely related to BTV, but does not match with any of the 24 serotypes of BTV. Hence, TOV is likely to represent a 25th serotype of BTV. Why TOV so far was only found mainly in Switzerland could be explained by the fact, that we employed an rRT-PCR protocol not widely used in other laboratories. Based on the sequence differences between BTV and TOV it could be shown that our rRT-PCR protocol was the only published method likely to detect TOV, whereas other commonly used methods failed to detect TOV due to numerous mismatches in the primer and probe regions. Parts of the viral RNA segment 2 from all TOV isolates were cloned and sequenced in order to (i) determine the genetic heterogeneity of TOV, and (ii) to identify conserved regions within S2 suitable for the design of rRT-PCR primers and probe for the detection of TOV and its differentiation from BTV. All TOV isolates showed an overall identity level of 95%. The fact that TOV is markedly different from BTV and shows a high level of sequence conservation among various isolates, suggests that TOV is not a reassortant or mutant of BTV but rather a different orbivirus that might have been circulating for a long time unnoticed, because it does not cause any clinical signs in goats (1). Based on the TOV-specific rRT-PCR and the newly available synthetic positive control for this assay, it will now be possible to look at the epidemiology, genetics, and pathogenesis of this virus in an extended way, even though TOV still cannot be propagated in any cell culture system so far.

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BLUETONGUE VIRUS IN NORTHERN AND WESTERN EUROPE: VACCINATION STRATEGIES AND PROTECTION IN SHEEP

Chris Oura[1], Toby Floyd[2], James Wood[2] and Carrie Batten[1]

[1] *Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey, UK*
[2] *Cambridge Infectious Diseases Consortium (CIDC), Cambridge University Vet School, Cambridge, UK*

Keywords: bluetongue, control, vaccination

Introduction
Bluetongue (BT) is a non-contagious Culicoides-borne viral disease affecting cattle, sheep and goats, as well as camelids and wild ruminants such as deer. The strain of BTV-8 currently active in northern Europe appears to be particularly virulent, and many clinical cases have been reported in cattle and goats. The virus is spread between hosts by biting midges of the genus *Culicoides*. Midges feeding on an infected animal, ingest virus along with a blood meal and the virus infects and replicates in the cells lining their gut wall. The virus then spreads through the body to the salivary glands, where it again replicates ready to infect the next susceptible animal that the midge feeds off. Although transmission by midges is by far the most common mechanism of transmission, evidence has recently been published that the northern European strain of BTV-8 can cross the placenta of an infected pregnant cow and infect the foetus, and also that it may be possible to infect cattle via the oral route.

Vaccination against BTV-8 in Northern Europe.
From 2006 to 2008 Bluetongue virus serotype 8 (BTV-8) has caused major economic losses in many countries across northern and western Europe. In 2008 the European Union agreed to support a mass-vaccination programme in all affected countries. Many pharmaceutical companies developed inactivated vaccines against BTV-8 which were brought onto the market throughout 2008. The first batch of 10 million doses of the BTV-8 inactivated vaccine selected for use in the UK came on the market in April 2008. These vaccines have been used extensively and effectively across countries affected by Bluetongue in Northern and Western Europe and outbreaks of BTV-8 reduced dramatically in 2008 in countries that vaccinated prior to circulation of the virus.

In the talk I will briefly review the epidemiology of BTV in Northern Europe and describe the incursions of different BTV serotypes into Europe up to August 2009. I will then describe the vaccine strategies used in different countries in Europe in 2008 with special reference to the vaccination programme carried out in Germany, France and the UK. I will discuss briefly the successes and failiures of the relevant campaigns**.**

The efficacy and length of protection of an inactivated BTV-8 vaccine: The BTV-8 inactivated vaccines used across Europe were produced very rapidly and were licenced for emergency use without any associated efficacy guaranties. Although many millions of animals in Europe have now been vaccinated across Europe, there remain some unanswered questions about the efficacy of the vaccines (ability to protect and reduce viraemia), the levels of seroconversion and neutralising antibodies in vaccinated animals, the length of the protective period, the safety of the vaccine in pregnant animals and both the extent and length of colostral antibody protection in lambs and calves born from vaccinated dams.

In this talk I will describe some BTV-8 challenge experiments carried out in the UK showing that a single dose of inactivated vaccine (Intervet, Bovilis BTV-8) against BTV-8 provided 100% protection against clinical disease caused by BTV-8 challenge and prevented virus replication in 6 out of the 7 sheep. There was a very strong anamnestic response in the vaccinated sheep on challenge and a good correlation between the presence of neutralising antibodies at the time of challenge and viral replication indicating that, if neutralising antibodies are present in the sheep at the time of challenge, they are likely to be protected from viral replication and clinical disease. These data indicate that sheep vaccinated on a single occasion with a BTV-8 inactivated vaccine are protected from clinical disease for at least 10 months after vaccination.

The extent and length of colostral antibody protection in lambs born from ewes vaccinated twice with an inactivated BTV-8 vaccine.
Key questions that farmers need addressing are:
1. What is the extent and length of colostral antibody protection in lambs born from vaccinated dams?
2. Are lambs born from BTV-8 vaccinated dams protected for up to 14 weeks - the time many lambs are kept prior to slaughter?
3. What is the optimal time at which lambs born from vaccinated dams should be vaccinated thus avoiding interference of the vaccine by colostral antibodies?

These questions need to be addressed for the following reasons:
1. If the duration of colostral antibody protection is shorter than the time taken for the lamb to reach slaughter weight it will be necessary to vaccinate the lambs as they will be alive during a high risk period when both Culicoides vector midges and BTV will be circulating (May - October).
2. To optimise the best time to vaccinate lambs it is essential to know at what point colostral antibodies will have sufficiently waned so they do not interfere with the vaccine. If there is high levels of interference produced from colostral antibodies it may be wise to recommend that lambs are vaccinated on 2 occasions.

I will describe a study that we have carried out in which we have challenged pregnant ewes sourced from the field which have been vaccinated on two occasions with the Intervet Bovilis BTV-8 inactivated vaccine, the first vaccine being given in May 2008 and the second vaccine given in November 2008, 4 weeks prior to lambing. The pregnant ewes and lambs in the group were blood tested at the time of lambing and the lambs were brought into the isolation units for challenge with BTV-8 when they were approximately 14 weeks old. Samples from the lambs were tested for antibodies by cELISA, for neutralising antibodies by a serum neutralisation assay (SNT) and for the presence of viral RNA by RT-PCR and for BTV by virus isolation at various time-points for 22 days post infection.

In this study we found that all the challenged lambs were protected from clinical disease at 12-14 weeks age and the majority of the lambs (80%) were also protected from viral replication. We also found that antibodies levels (measured by both ELISA and SNT) did not increase on viral challenge suggesting that there was some level of interference in these 14 week old lambs. However we were not able to rechallenge the animals after colostral antibodies had waned to see if they remained protected from disease.

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DETECTION OF BLUETONGUE SEROTYPE 1 IN RED DEER (*CERVUS ELAPHUS*) BY RT-PCR

Belén RODRÍGUEZ-SÁNCHEZ[1], Christian GORTÁZAR[2], Francisco RUIZ-FONS[3], José M. SÁNCHEZ-VIZCAÍNO[1]

[1]Animal Health Department, Veterinary Faculty, Universidad Complutense de Madrid, Spain;
[2]Instituto de Investigación en Recursos Cinegéticos IREC (CSIC – UCLM – JCCM), Ciudad Real, Spain;
[3]NEIKER- Instituto Vasco de Investigación y Desarrollo Agrario, Department of Animal Health, Derio, Bizkaia, Spain

Keywords: antibodies, bluetongue, RT-PCR, wild ruminants

Introduction and objectives

Bluetongue (BT) is a vector-borne disease caused by a virus belonging to the Orbivirus genus. Sheep are considered the most vulnerable species for BT, but other ruminants are known to play an important role as reservoir of the virus. This is the case of cattle, used as sentinels in surveillance programs, although cattle also displays important BTV symptoms when infected with BTV-8, as shown in the epidemic caused by this serotype in Central Europe (1). The role of wild ruminants in the spreading and persistence of the virus has only started to be elucidated. Several studies have reported the presence of either BTV antibodies (2, 3) or the virus in red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), mouflons (*Ovis aries*), and several other wild bovids and cervids (2). The presence of BTV and BTV-specific antibodies in wild species underscores the importance of these species due to fact that, except for mouflons, these species are asymptomatic hosts. Moreover, although their viraemia is generally considered to be short -16 days- it may resume in response to stress (4). This, together with their capacity to overcome the infection and free-range live, makes them a suitable factor for virus spreading. Besides, wild animals could also be involved in the mechanism of overwintering, which allows the virus to survive in a latent form during the vector-free season (5).

The main focus of this study was to carry out a molecular survey on deer from the Southern extreme of the Spanish mainland in order to evaluate the sanitary status of the deer population regarding BT and analyze its potential role as BT sentinels. The studied area is especially important from an epidemiological point of view due to the fact of being located within the distance reached by the winds that blow from the North of Africa to the South-West of Spain. These winds can carry, together with sand particles, *Culicoides* midges, known to be the arthropod vector for BTV (6). The susceptibility of wildlife in this area to BT infection will provide valuable information about its role in the spreading and circulation of different BTV serotypes.

Materials and methods

In order to analyze the sanitary situation of wild ruminants in Spain immediately after the occurrence of BTV-1,a highly pathogenic serotype, blood and serum samples were collected from 510 red deer in a site with known occurrence of BTV among domestic ruminants. Blood samples were collected in EDTA sterile tubes by cervical puncture and frozen at -20ºC. All samples were obtained from farmed red deer. Thus, the exact age was known for each individual. Samples from adult deer (n=160) were taken in July, and samples from yearling deer (n=350) were taken in August 2007. 100 µl of each blood sample were used for RNA extraction using a commercial kit (Macherey-Nagel) based on silica-gel columns. Four different RT-PCR tests were applied to the samples: 1) A group-specific RT-PCR for the detection of a conserved region within the NS1 segment (7) and 2) a serotype-specific RT-PCR that detects BTV-1 (8). Each sample was tested in duplicates. Both assays were carried out as a conventional, one-step RT-PCR.

Results

Samples were divided into adult animals (160 samples) and yearlings (350 samples). 25% of the adults were positive to the BTV group-specific RT-PCR assay. Positive samples were sequenced in order to confirm the presence of BTV and further analyzed for the identification of the serotype. None of the samples from adult deer were positive to BTV-1 specific RT-PCR.

Yearling samples, however, gave a different pattern of results: 16.33% of the animals were positive both to the group-specific and to the BTV-1 specific RT-PCR assay. All the amplified products were sequenced and the presence of BTV (group-specific sequence) and BTV-1 were confirmed using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Discussion and conclusions

We hypothesized that if deer were the optimal sentinels for BT, the virus or specific antibodies would be detected among these even earlier than among sentinel cattle from the same geographic area.

What has been observed in this study is in accordance with the epidemiological situation of Spain at the time the samples were collected: adult deer were sampled on the 12th of July 2007, whilst the yearlings were sampled on the 20th of August 2007, i.e, 26 days after BTV-1 was notified in the South of Spain, 50 km from the deer farm where samples were collected. Thus, adult deer were sampled when BTV-1 was not present in the country yet, which supports the lack of detection of BTV-1 in adult animals. However, 25% of the adult deer were positive to the BTV group-specific RT-PCR test, which means that they could be asymptomatic hosts for BTV-4 or that we failed in the detection of BTV-1 in these samples.

On the other hand, yearlings were already positive to BTV-1 only 26 days after this serotype was first reported in livestock in the same area. No data has been collected from deer samples prior to the report of BTV-1 in livestock, but, preliminarily, wild ruminants could make excellent sentinels for BT in the studied area.

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EXPERIMENTAL INFECTION OF RED DEER (*CERVUS ELAPHUS*) WITH BLUETONGUE VIRUS SEROTYPES 1 AND 8

Jorge Ramón López-Olvera[1], Caterina Falconi[2], Paloma Férnandez-Pacheco[3], Jovita Fernández-Pinero[3], Miguel Ángel Sánchez[3], Agustín Palma[3], Irene Herruzo[3], Joaquín Vicente[2], Miguel Ángel Jiménez-clavero[3], Marisa Arias[3], José Manuel Sánchez-Vizcaíno[4], Christian Gortázar[2]

[1]*Servei d'Ecopatologia de Fauna Salvatge (SEFaS), Universitat Autònoma de Barcelona (UAB), Bellaterra, Barcelona, Spain;*
[2]*Instituto de Investigación en Recursos Cinegéticos (IREC; CSIC-UCLM-JCCM), Ciudad Real, Spain;*
[3]*Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, Madrid, Spain;*
[4]*Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid (UCM), Madrid, Spain*

Keywords: bluetongue; red deer; reservoirs, wildlife

Introduction and objectives

Bluetongue (BT) is an emerging infectious disease in Europe. Bluetongue virus (BTV) serotypes of concern in Europe include BTV-1, expanding northwards since its first introduction in Southern Spain in 2007, and BTV-8, spreading throughout Europe since its introduction in The Netherlands in 2006. Vertical transmission of the disease and overwintering even in the northernmost areas of this continent have been reported for BTV-8. BTV-8 reached the Iberian Peninsula in 2007, where since then it coexists with BTV-1 in some regions. Vaccination of domestic ruminants is taking place throughout Europe to control BT expansion. A high prevalence of serum antibodies against BT has been reported in red deer (*Cervus elaphus*) in Southern Spain (Ruiz-Fons et al. 2008, García et al. 2009), but its potential role as a reservoir for domestic livestock is still unknown. In North American white-tailed deer (*Odocoileus virginianus*), experimental infection with BTV-8 caused mortality in fawns, whereas adult deer developed viraemia but limited clinical signs for ten days after exposure (Vosdingh et al. 1968). However, the potential role of European wild ungulates as reservoirs of BTV has not been assessed, in spite of antibodies detection in red deer in this continent (Linden et al. 2008) and sporadic fatal disease with virus isolation in mouflon (*Ovis aries*) (Fernandez-Pacheco et al. 2008).

Materials and methods

Eleven seven month-old Iberian red deer females were transported into the Centro de Investigación en Sanidad Animal (CISA) facilities in Valdeolmos (Madrid) on January 19th 2009. On January 26th (0 days post-infection or dpi) four of the deer were inoculated intravenously (i.v.) with $2{,}5x10^6$ $TCID_{50}$ bluetongue virus serotype 1 (BTV-1), four were inoculated i.v. with $2{,}5x10^6$ $TCID_{50}$ BTV-8, and the three remaining deer received an equivalent volume of cell culture medium by the same way, acting as controls.

The deer were monitored daily from 0 dpi to 12 dpi and on days 14, 17, 21, 24, 28, 31, 38, 50, 60, 66, 71, and 78 dpi. Monitoring included exploration for clinical signs of bluetongue (including fever, tongue and oral mucosa lesions, conjunctivitis, oedema, hoof lesions), as well as collection of blood samples with anticoagulantfor real time RT-PCR analysis and without anticoagulant for serum. Deer were euthanized on 98 (four deer), 105 (four deer), and 112 dpi (three deer).

Viral load in blood was assessed by semi-quantitative real-time RT-PCR as described (Toussaint et al, 2006), using an internal control (β–actin). Serum antibodies were analyzed by commercial ELISA and virus-neutralization test (VNT).

Results

No clinical signs of BTV were observed in any deer. All the deer inoculated with BTV-1 and three out of the four deer inoculated with BTV-8 became viraemic. Virus RNA was detected by real time RT-PCR from 1 dpi to the end of the study in these deer, with a peak around day 12 after inoculation in both serotypes, and a slow decline thereafter. One of the deer inoculated with BTV-8 failed to develop a stable viraemia: only low amounts of virus RNA were detected until 14 dpi, and it remained PCR-negative for the remaining of the study period.

Serum antibodies against BTV were detected in all inoculated deer both by virus-neutralization and ELISA tests. In deer inoculated with BTV-1, ELISA revealed BTV-specific antibodies by 10 dpi, and the neutralizing antibody response was first detected at 8-11 dpi, with peak titres of 1/1280 around 17-21 dpi. In deer inoculated with BTV-8, ELISA revealed BTV-specific antibodies between 9 and 12 dpi, whereas neutralizing antibodies appeared slightly earlier (8 dpi) with peak titres of 1/640 to 1/1280 between 17 and 28 dpi. BTV-specific antibodies remained present throughout the whole study period.

No virus RNA was detected in the control deer until 38 dpi. However, on 38 dpi one control deer, which shared box with one BTV-1 and the low-viraemic BTV-8 inoculated deer, developed BTV-1 viraemia, followed by seroconversion between 38 and 50 dpi.

Discussion and conclusions

This study demonstrates that BTV serotypes 1 and 8 are able to replicate in Iberian red deer. Both serotypes are currently affecting livestock throughout Europe, but this is the first time the susceptibility of a European wild ungulate is shown. Our results confirm that Iberian red deer get infected and develop an intense, asymptomatic, and long-lasting viraemia, at least under the experimental conditions tested. Red deer is an abundant wild ruminant in many parts of the northern hemisphere, occurring in BTV affected regions of central and southern Europe. Therefore, red deer could play a role in the still unclear epidemiology of BTV in this continent.

Transmission of BTV in absence of the vector has been previously reported both in domestic and wild ungulates (Vosdingh et al. 1968, Backx et al. 2009), as it happened with the spontaneously-infected red deer of our study. Both wound contact and oral transmission have been suggested to explain it, but further experiments should be carried out to elucidate that point.

Further studies on the vector-host interface are ongoing to evaluate the role of red deer as a reservoir species for BTV in Europe.

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INFECTION DYNAMICS OF THE CURRENT NOVEL 'SWINE-LIKE' HUMAN INFLUENZA A/H1N1 IN PIGS: A/CALIFORNIA/07/09

Sharon BROOKES[1], Alejandro NUNEZ[1], Derek CLIFFORD[1], Steve ESSEN[1], Richard IRVINE[1], Kristien VAN REETH[2], Gaelle KUNTZ-SIMON[3], Willie LOEFFEN[4], Emanuela FONI[5], Lars LARSEN[6], Mikhail MATROSOVICH[7], Michel BUBLOT[8], Jaime GARCIA[9], Martin BEER[10], Giovanni CATTOLI[11], Ian BROWN[1]

[1]Veterinary Laboratories Agency-Weybridge, Addlestone, Surrey, KT15 3NB, UK; [2]University of Ghent, Salisburylaan 133, 9820 Merelbeke, Belgium; [3]AFSSA, 27-31 avenue du Général Leclerc, 94701 MAISONS-ALFORT Cedex, France; [4]Central Veterinary Institute, Lelystad, P.O. Box 65 8200 AB Lelystad, The Netherlands; [5]Istituto Zooprofilattico Sperimentale Lombardia ed Emilia Romagna, Sezione di Parma, Via dei Mercati 13 A, 43100 Parma, Italy; [6]National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, Building 11, room 1.118, 1790 København V, Denmark; [7]Institute of Virology, Philips University, Hans-Meerwein-Strasse 2 DE-35043, Marburg, Germany; [8]Merial, 29, avenue Tony Garnier, 69007 Lyon, France; [9]Laboratorios HIPRA, Avda. La Selva, 135, 17170 AMER GIRONA, Spain; [10]Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Suedufer 10, D-17493 Greifswald - Insel Riems, Germany; [11]IZSL Padova, Viale dell'Università, n. 10 – 35020, Legnaro (PADOVA), Italy

Key words: novel influenza A/H1N1, pigs

Introduction and objectives

Since the emergence of the novel swine-like human influenza A/H1N1 virus in April 2009 there have been in excess of 15,000 confirmed cases in people including 99 mortalities. Whilst over 90% of cases have been reported from the Americas, there has been a rapid global dissemination (48 countries reporting cases; PAHO, 2009) and on-going concerns in relation to pandemic potential also remain high (WHO level 5) particularly as the seasonal human influenza period approaches the Northern hemisphere. In addition, this virus has already demonstrated the property of reverse zoonosis (Irvine and Brown, 2009) crossing the species barrier under natural conditions from a person to pigs in a single herd in Alberta, Canada. Exposed animals had mild disease and recovered (OIE, 2009).

The objective of our study was to investigate infection dynamics, clinical outcome, pathogenesis, host susceptibility and transmissibility of the current novel H1N1 human influenza A virus in pigs.

Materials and methods

The virus strain (A/California/07/09) was propagated in embryonated fowls' eggs and animals were inoculated intra-nasally using a mucosal atomisation device (MAD®) to mimic aerogenous infection. The total dose was $10^{5.8}$ EID_{50} delivered in a final volume of 2ml per nostril.

Landrace hybrid pigs were sourced from a high health status herd. All pigs were 4-5 weeks of age (weight 5-8 kg) at the start of the study and were shown to be both influenza A virus and antibody negative by RRT-PCR and HI assays. The 22 animals were randomly allocated to study groups – infected (11), contacts (8), controls (1) and mock-infected (2).

Post-mortems examinations were carried out on infected animals at dpi 1, 2, 3, 4, 7 and 21 (n=2, except at dpi1, n=1). Infected animals were swabbed (nasal-N, oral-OR, ocular-OC, rectal-R) daily with material processed for Matrix (M) gene RRT-PCR, and were bled daily from dpi 1-4, then twice weekly for HI testing (serum) and haematology (EDTA).

Transmission dynamics were initially established by placing two contact pigs in the same room as the infected pigs at dpi 2, which remained in contact until virus shedding in both contacts was established (~72 hrs). The first two contact pigs were then removed to a separate room and two more naïve pigs added (2+2) until shedding was established. This process was repeated for up to four cycles of transmission. All contact animals were swabbed and assayed daily as above.

Results

All inoculated animals became infected and shed virus mainly via the nasal route from dpi 1-9; peak shedding occurred between dpi 3-5. Oral and ocular shedding was detected, but rectal shedding was not proven. Shedding had ceased by dpi 10. Pyrexia (>39.5ºC) was detected from dpi 1-6 with variable peaks and some biphasic profiles. Weight loss was limited (~5%, between dpi 6-8) and clinical scores peaked at dpi 4-6 with progressive recovery evident (n=2) after dpi 7. Clinical signs included nasal discharge, respiratory signs (cough mainly, some increased respiratory rate), ocular discharge, lethargy and inappetence. There were no mortalities. Minor focal pathological lesions were observed in the apical lobes of the lung at dpi 2-4, and at dpi 7 more extensive gross lesions were observed.

The contact animals developed similar profiles to those above, particularly after transmission cycles one and two (72 hr contact period). Contact animals became infected by one day post-contact (dpc), based on RRT-PCR shedding profiles, with transmission cycle 1 shedding peak at dpc 3, cycle 2 peak at dpc 5/6, with shedding ceased at dpc 6-9. Pyrexia was evident from dpc 5-9. Clinical signs appeared milder than in those directly infected. Transmission cycles three and four were substantially delayed (third cycle >4 days).

Full data sets of virus shedding, systemic dissemination of virus, pathological characteristics of infection, humoral responses, sequence variation and selected immunological correlates of infection will be presented.

Discussion and conclusion

Swine are susceptible to, and clinical disease is induced following infection with the newly reported novel swine-like human influenza A/H1N1 virus. Importantly, infected animals are able to transmit the virus to naïve contact animals successively for at least three cycles of transmission.

Our data provides a robust scientific evidence base for both veterinary and public health risks associated with these novel A/H1N1 viruses. Reverse zoonosis has occurred naturally and we have now demonstrated this experimentally. The latter has important implications for potential reassortment events, not only in humans, but also in swine, and thus the potential for the generation of hazardous variants.

There was no mortality in pigs based on preliminary data. However, the levels of morbidity and the role of other disease and/or husbandry factors would result in economic impacts to the swine industry.

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CONTACT EXPOSURE EXPERIMENTS USING THE NEW INFLUENZA A/H1N1 VIRUS

Elke LANGE[1], Donata KALTHOFF[2], Ulrike BLOHM[1], Timm HARDER[2], Jens TEIFKE[1], Angele BREITHAUPT[1], Christina MARESCH[1], Thomas METTENLEITER[3], Martin BEER[2], Thomas VAHLENKAMP[1]

[1]Institute of Infectology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany;
[2]Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany;
[3]Institute of Molecular Biology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany;

Keywords: chicken, Influenza A/H1N1, pig

Introduction and objectives

Influenza A/H1N1 viruses were first isolated from swine in 1930 and have been shown to be antigenetically highly similar to a recently reconstructed human 1918 influenza A/H1N1 virus. From the 1930 to the late 1990s these classical swine influenza viruses circulated in the US in swine and remained relatively antigenetically stable. The relative antigenic stasis of classical influenza A/H1N1 viruses in swine until 1998 during the time when siginificant antigenic drift of influena H1 viruses in humans was observed has created a substantial antigenic gap between classical swine and human seasonal H1 viruses. Thus, swine have become a reservoir of influenza H1 viruses with the potential to cause significant respiratory disease or even a possible pandemic in humans.

Since its identification in April 2009 an influenza A/H1N1 virus containing a unique combination of gene segments from both North American and Eurasian swine lineages has continued to circulate in humans. Homology analyses between the 2009 influenza A/H1N1 virus and its nearest relatives indicated that its gene segments may have been circulating undetected for an extended period. As of May 29th 2009, there have been 15510 laboratory confirmed cases in 53 countries, resulting in 99 deaths. A key determinant of the current influenza in humans is the transmission rate of the new influenza A/H1N1 virus.

The objectives of the current studies were to investigate (i) whether experimental intranasal infection in pigs with the 'new' influenza A/H1N1 virus results in clinical signs and whether the infection would be transmitted to naïve contact pigs.

Materials and methods

Six pigs at the age of 10 weeks were infected intranasally with 10^6 $TCID_{50}$ of the new influenza A/H1N1 virus in the high containment facilities at the Friedrich-Loeffler-Institut, Germany. From day one post infection (p.i.) 3 naïve pigs and 5 naïve chicken were housed together with the infected animals as contact animals. All animals were monitored for virus excretion and H1 specific antibodies.

Oropharyngeal swab samples were taken daily from the infected and contact pigs and cloacal as well as oropharyngeal swab samples were collected daily from the chicken. EDTA blood samples were collected from the pigs before the infection and on day 1, 2, 3, 5, 7, 10, and 14 p.i. Virus excretion was investigated by real time PCR and virus isolation. The anti-influenza immune response was monitored by haemagglutination inhibition test and by ELISA. One experimentally infected pig was euthanized on day 2 p.i. for necropsy.

Results

Experimentally infected pigs developed clinical symptoms from day 3 p.i. onwards including nasal discharge, sneezing, salivation, diarrhea, fever, emaciation, lid edema and/or compromised general condition. None of the animals displayed more than 4 of the clinical symptoms at one time and clinical signs were mild. Most prominent was diarrhea which developed between day 3 and 7 p.i. Nasal discharge and/or sneezing was mainly observed between day 4 and 5 p.i. Animals excreted virus and developed an anti-influenza virus immune response.

All three naïve contact pigs also developed similar clinical signs with some delay. Virus excretion and the development of anti-influenza antibodies were observed.

Immunological analysis revealed an early activation of $CD4^+$ T-cells and a transient increase in $CD4^+$ and $CD8^+$ T-cells as well as B-cells between day 5 and 6 p.i.

The infected animal euthanized on day 2 p.i. did not show any lung lesions, except a slight hyperemia of the nasal turbinates.

The 5 naïve chicken did not develop clinical signs, did not excrete virus and also did not develop anti-influenza virus antibodies.

Discussion and conclusions

The current investigation showed that experimental intranasal infection of pigs with 10^6 $TCID_{50}$ of the new influenza A/H1N1 virus results in clinical signs. Diarrhea was prominent among the infected group of animals between day 3 and 7 p.i. Typical influenza-like symptoms like sneezing and nasal discharge were observed between day 4 and 5 p.i. which is somewhat delayed compared with former experimental infection experiments using porcine influenza A/H1N1 viruses. Infected pigs excreted virus and transmitted the infection to naïve contact pigs which displayed similar clinical signs and also developed an anti-influenza immune response.

In contrast to the transmission of the infection to contact pigs, no infection of contact chicken occurred. Obviously the high transmissibility of the virus observed in humans also applies to pigs but not for the transfer of the infection from pigs to chicken. After final analysis of the data it remains to be investigated whether other bird and mammal species are susceptible to the infection with this newly discovered influenza virus.

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GEOGRAPHIC SPREAD OF AN ENCEPHALITIC LINEAGE 2 WEST NILE VIRUS STRAIN IN 2008 IN CENTRAL EUROPE

Tamás BAKONYI[1,2], Károly ERDÉLYI[3], Emőke FERENCZI[4], Orsolya KUTASI[5], Bernhard SEIDEL[6], Herbert WEISSENBÖCK[7], Norbert NOWOTNY[2]

[1]Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Budapest, Hungary;
[2]Zoonoses and Emerging Infections Group, Clinical Virology, Department of Pathobiology, University of Veterinary Medicine, Vienna, Austria;
[4]Department of Virology, National Center for Epidemiology, Budapest, Hungary;
[3]Central Veterinary Institute, Budapest, Hungary;
[5]Large Animal Clinic, Faculty of Veterinary Science, Szent István University, Üllő, Hungar;
[6]Office of Ecology Research and Landscape Assessment, Persenbeug, Austria;
[7]Institute of Pathology and Forensic Veterinary Medicine, Department of Pathobiology, University of Veterinary Medicine, Vienna, Austria

Keywords: arbovirus, encephalitis, horse, human, lineage 2, mosquito, West Nile fever, wild bird

Introduction and objectives

An exotic, lineage 2 West Nile virus (WNV) strain emerged in 2004 in Hungary, and caused sporadic, fatal encephalitis in goshawks (*Accipiter gentilis*) (1). The same strain was detected in 2005 in encephalitic birds of pray and in a sheep, at the same geographic region of the country (South-East) (2). While in 2006 WNV infections were not detected in the previously affected populations, in 2007 the lineage 2 strain re-emerged in the same geographic region and caused lethal encephalitis cases in geese, in birds of pray, and also in a horse. In this presentation our observations on the WNV activity in 2008 in Hungary and in Austria is reviewed.

Materials and methods

Wild birds succumbed in central nervous system (CNS) symptoms were submitted for laboratory investigations by the officers of BirdLife Hungary, and of National Parks, as well as by falconers. Serum and cerebrospinal fluid (CSF) samples from human encephalitic cases were sent to the national reference laboratory by physicians, hospitals and epidemiologists. Serum and EDTA-blood, CSF, and tissue samples of horses suffering or succumbed in CNS symptoms were received for diagnostic investigations from veterinarians. Limited number of mosquitoes was collected in a red-footed falcon (*Falco vespertinus*) colony nesting place, where WNV-induced bird mortality was recorded in 2007. In Austria wild bird samples were collected by the BirdLife Austria, and samples of captive raptors were sent by falconers. Mosquito samples were collected in four federal states of Austria in WNV affected and non-affected regions.

Direct virus detection methods were based on reverse-transcription polymerase chain reaction (RT-PCR) using universal flavivirus-, and WNV lineage 2 specific primers, TaqMan real-time RT-PCR using WNV lineage 2 specific primers and probe, and immunohistochemistry. Positive samples were subjected to virus isolation on Vero cells, chicken embryo and in suckling mouse brain.

Viruses were identified by partial genome sequence determinations and sequence comparisons.

Serological investigations were performed on human, horse and wild bird samples. WNV specific IgG and IgM antibodies were detected by indirect immunofluorescence assay (IFA), haemagglutination inhibition test (HIT), plaque reduction microneutralization test (PRμNT), and competitive ELISA.

Results

In Hungary the first WNV positive wild bird samples in 2008 were identified in the middle of August. Predominantly goshawks succumbed in WNV encephalitis (15 diagnosed cases), but viral nucleic acid was also demonstrated in two Harris hawk (*Parabuteo unicinctus*) and in three Gryfalcon (*Falco rusticolus*) samples. The samples were originated mainly from the central and western part of the country. In the beginning of September WNV nucleic acid and antigens were detected in goshawks, which were found dead in Lower-Austria, close to Vienna. A few weeks later the virus was also detected in other captive Austrian wild birds, including a Kea (*Nestor notabilis*) and a Bearded Vulture (*Gypaetus barbatus*). WNV was isolated from several bird samples.

The number of reports on horses with CNS symptoms increased in August and in September in Hungary. Symptomatic horses (n: 30) were tested for WNV by direct and indirect methods. WNV nucleic acid was detected in one horse, while WNV-specific antibodies were found in 16 horses' samples (IFA titres between 1:80 and 1:2560). Subsequently, asymptomatic horses (n: 86), which were kept in the same perishes as the sick ones, were also surveyed serologically by ELISA, and 29 of them was found positive.

The National Center for Epidemiology reported 14 serologically confirmed human WNV cases in Hungary between August and October, 2008 (3). Patients were diagnosed with meningitis and encephalitis. Positive cases were found in every regions of the country.

The distribution of WNV in mosquito vectors was surveyed in the eastern region of Austria. Mosquito pools (n: 138) were tested by real-time RT-PCR. Six pools of adult *Culex pipiens* mosquitoes were found positive. In Hungary, one *Cx. pipiens* pool was found positive out of 12 investigated pools.

The partial nucleotide sequences of the detected viruses were >99% identical with the WNV lineage 2 strain, which was first identified in 2004 in Hungary.

Discussions and conclusions

In 2008 considerable increase and geographic spread of the previously detected, encephalitic WNV lineage 2 strain was observed in Hungary. The virus reached Austria and caused the first documented cases in the country. The favourable weather conditions might have supported the increase in virus activity; however, so far unidentified avian host are suspected to play a role in the spread of the virus. The goshawk mortality was an early indicator of the epizootic. Surveillance and monitoring systems for the early detection of cases in the 2009 epizootic season, as well as immunization of susceptible horse populations are needed.

Acknowledgements

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CHARACTERIZATION OF THE EQUINE WEST NILE VIRUS OUTBREAK IN HUNGARY IN 2008

Sylvie LECOLLINET[1], Orsolya KUTASI[2], Sara SARDI[2], Imre BIKSI[2], Céline BAHUON[1], Tamás BAKONYI[3], Stéphan ZIENTARA[1]

[1]AFSSA, UMR 1161 Virologie, Maisons-Alfort, France;
[2]Faculty of Veterinary Science, Large Animal Clinic, Budapest, Hungary;
[3] Faculty of Veterinary Science, Dept. of Microbiology and Infectious Diseases, Budapest, Hungary

Keywords: encephalomyelitis, lineage 2, West Nile virus

Introduction and objectives
In 2008, West Nile Virus (WNV) has been responsible for neurological conditions in humans and/or horses and birds in Romania, Italy and Hungary and has been associated with wild bird deaths in Austria. In Hungary, WNV infections have resulted in 14 neuro-invasive cases in humans and in the death of multiple birds of prey.

Materials and methods
We investigated 26 equids showing neurological disorders between August and December 2008. Clinical and virological data were collected. Antibody responses, either IgM, IgG or neutralizing responses, were evaluated following OIE guidelines, by ELISA assays or PRNT90 test. In an attempt to characterize the virus, isolation, nested RT-PCR and sequencing techniques were used. Histopathological examination was carried out on 4 horses.

Results
Serological investigations revealed positive IgG and IgM antibody responses in 18 horses, confirming that neurological condition in those horses were due to recent WNV infection. Neutralizing antibody titers were comprised between 10 and 270 (median 94±72) 2-35 days after the onset of clinical signs. The main symptoms displayed by affected horses were ataxia and weakness. Assymetric gait abnormalities, muscle tremors, hypersensitivity, cranial nerve deficits and recumbency were also common signs. Thirteen animals recovered within 2 months. The neuropathological pattern was characterized by lymphocytic-plasmocytic perivascular infiltration and gliosis. One from ten horses tested positive for WNV in their leukocytes.

Two horses were also assessed for the presence of WNV in their nervous system; both specimens were positive and allowed for molecular characterization of the viral strain. The phylogenetic tree, which has been drawn after multiple alignment with diverse lineage1 and 2 strains, clearly shows that the currently circulating WNV belongs to lineage2 and has slightly diverged from the hungarian virus isolated in 2004 (see figure 1).

Discussion and conclusions
This equine WNV outbreak in Hungary has been caused by a lineage2 virus, often considered as non pathogenic for animal or human species. Lineage2 virus had been shown to circulate in Hungary and caused isolated encephalomyelitis cases in different species, but had never been associated with important equine outbreak. According to the localisation of the equine and avian cases Hungary experienced great north-western spread of the pathogen.

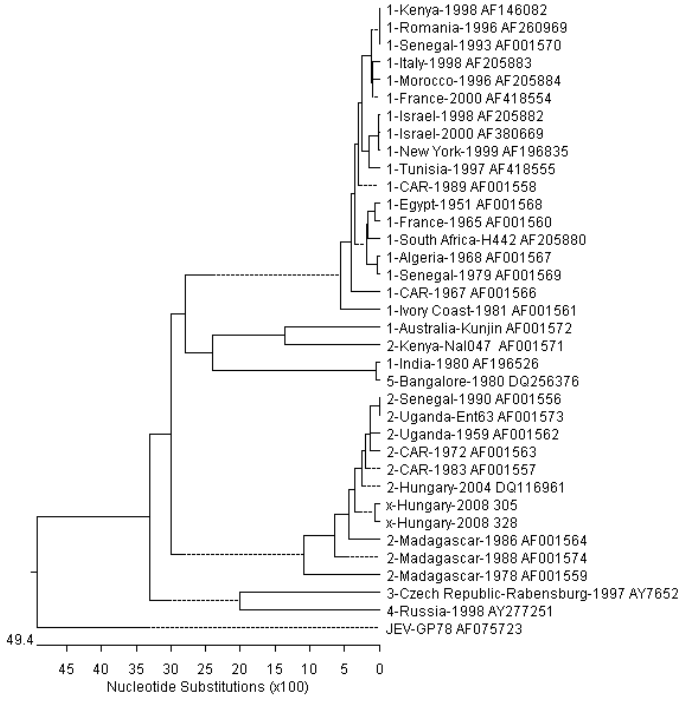

Figure 1. Phylogenetic tree based on the sequencing of 254nt of the envelop gene of WNV viruses belonging to diverse lineages (lineages 1 to 5, as proposed by Bondre et al.). Sequences were named according to the following rule: lineage number-country -year and Genbank accession number.Sequences obtained from brain or spinal cord specimens of two Hungarian horses are indicated as x-Hungary- 2008 and are classified among lineage 2 viruses. Legend: CAR = Central African Republic, JEV = Japanese Encephalitis Virus.

NEWCASTLE DISEASE VIRUS IN WEST AFRICA: NEW VIRULENT STRAINS IDENTIFIED IN NON-COMMERCIAL FARMS

Chantal J. SNOECK[1], Mariette F. DUCATEZ[1], Ademola A. OWOADE[2], Olufemi O. FALEKE[3], Bello R. ALKALI[4], Marc C. TAHITA[5], Zekiba TARNAGDA[5], Jean-Bosco OUEDRAOGO[5], Issoufou MAIKANO[6], Patrick Okwen MBAH[7], Jacques R. KREMER[1], Claude P. MULLER[1]

[1]Institute of Immunology, National Public Health Laboratory, Luxembourg; [2]Department of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria; [3]Department of Public Health and Animal Production, Faculty of Veterinary Medicine, Usman Danfodio University, Sokoto, Nigeria; [4]Department Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Sokoto, Nigeria; [5]Institut de Recherche en Sciences de la Santé, Bobo-Dioulasso, Burkina Faso ; [6]Direction des Laboratoires Vétérinaires, Niamey, Niger ; [7]District Hospital Bali, North West Provincial Delegation of Public Health, Cameroon

Keywords: enzootic, Newcastle disease, phylogeny, virulent, West Africa

Introduction and objectives

Before the H5N1 Avian influenza virus outbreaks, NDV was considered as the most important virus in poultry. Depending on virus strains, it is responsible for mild to severe clinical signs that can result in severe economical losses in poultry farms and that are usually impossible to differentiate from symptoms induced by Avian influenza virus [2]. Although the poultry industry is important for local and national economies in West African countries, NDV was never phylogenetically characterized in this region.

Materials and methods

Between 2002 and 2007, tissues samples, cloacal, pharyngeal and tracheal swabs were collected in backyard farms, commercial farms, slaughterhouses and live bird markets in Nigeria, Niger, Burkina Faso and Cameroon. Most samples were from chickens but some were also obtained from turkeys, ducks, a fowl, a pigeon and a parrot. RNA was extracted (QIAamp Viral RNA Mini Kit, Qiagen; or MagMAXTM-96 AI/ND Viral RNA Isolation Kit, Ambion) and reverse transcripted. NDV positive specimens were detected as 280 or 550 bp fragments of the fusion protein gene (F gene) by nested PCRs, using previously published gene-specific primers. After purification, PCR products were sequenced in both orientations using nested PCR primers. Nucleotide sequences were determined on an ABI 3130 Avant capillary sequencer (Applied Biosystems).

Results

A total of 44 partial F gene sequences were phylogenetically compared with representatives of the six known genetic lineages of NDV circulating worldwide [1] and clustered in 5 different lineages. Lineage 2 viruses were genetically identical or similar to the locally used LaSota vaccine strain and were mostly detected in commercial farms (Fig. 1). Lineage 1 strains were related to the asymptomatic enteric Queensland V4/66 vaccine strain, but to the best of our knowledge no lineage 1 vaccine is currently used in West Africa, and the single lineage 3 strain was genetically similar to the Mukteswar vaccine strain, which is mainly used in South East Asia [3], suggesting that the corresponding viruses are wild-types. Sublineage 4b viruses are antigenically distinguishable from all other NDV lineages and are generally referred to as pigeon paramyxoviruses-1 (PPMV-1) [2]. They are mainly found in the Middle East, Asia and Europe [2] and it is not clear whether these viruses circulate in the country or whether they had been imported by bird trade. Twenty-one strains from backyard farms and live bird markets clustered in three different subgroups (Fig. 1) clearly distinct from any of the existing sublineage 5 groups, and were therefore considered as new sublineages, tentatively named 5f, 5g and 5h. All of these strains were predicted to be virulent on the basis of their F protein cleavage site sequence (RRQKR*F or RRRKR*F). The intra- and intersublineage diversity of established and new sublineage 5 groups has been assessed after selecting representative strains of each sublineage. The minimal genetic distances between the most closely related strains of the known sublineages 5a to 5e ranged from 2.3% (5a and 5c) to 9.2% (5b and 5d). In contrast minimal distances between the latter and new sublineages ranged from 9.4% (5a and 5h) to 15.9% (between 5e and 5g). The minimal distances between the new sublineages 5f to 5h ranged from 11.5% (5f and 5h) and 17.3% (5g and 5h).

Discussion and conclusions

Several lines of arguments suggest that lineage 5 strains have been enzootically circulating for many years in West Africa and have apparently not been exported to other continent: (1) the high genetic diversity of lineage 5 strains in West Africa, (2) their common ancestry suggested by the phylogenetic tree, (3) their presence in three different Sub-Saharan countries as well as (4) the absence of similar NDV strains on other continents. While lineage 5 seems to be the indigenous NDV variant to West Africa in the local breeds of backyard farms and possibly in wild birds that could be a wild reservoir for these enzootic strains, the other lineages seem to have different geographic origins including widely used vaccines. The absence of lineage 5 strains in commercial farms suggests that current vaccination programs efficiently protect commercial poultry. In conclusion, this study shows that as geographic surveys become more complete the genetic diversity of NDV seems to further unfold.

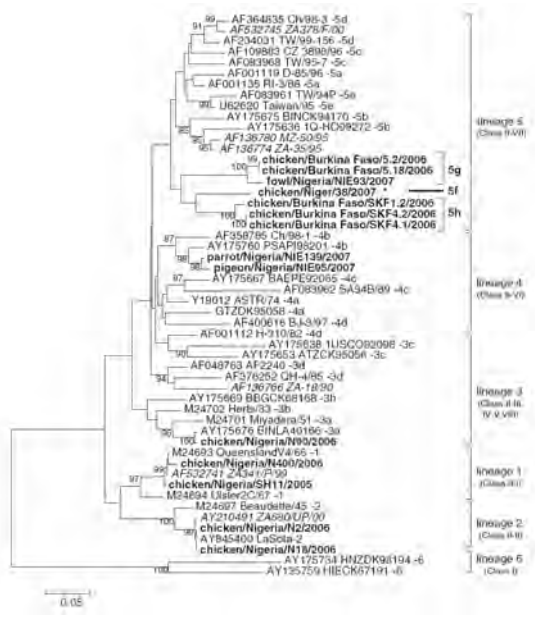

*Fig. 1. Phylogenetic analysis of 14 partial F gene sequences (in bold) based on nucleotides 67 to 420. The tree was constructed with the Neighbour-Joining method (Kimura-2 parameter, Mega v3.1) with 1000 bootstrap replicates. Only bootstrap values higher than 79 are shown. * The first 27 nt of the corresponding sequence are missing for this isolate. Strains from South Africa (ZA) and Mozambique (MZ) are shown in italic.*

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RECOMBINANT NEWCASTLE DISEASE VIRUSES AS MARKER VACCINES AGAINST HIGHLY PATHOGENIC AVIAN INFLUENZA

Angela RÖMER-OBERDÖRFER, Eylin TOPFSTEDT, Christian GRUND, Daniela DECKERS, Miriam RUDOLF, Jutta VEITS, Thomas C. METTENLEITER

Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany

Keywords: avian influenza, marker vaccine, recombinant Newcastle disease virus

Introduction and objectives

Newcastle disease virus (NDV) and highly pathogenic avian influenza virus (HPAIV) are two of the most important avian viruses which can cause severe disease in poultry, resulting in high economical losses for poultry industries. HPAIV caused infections in many Asian, European and African countries. Vaccine preparations against HPAIV consisting of inactivated whole virus mixed with different adjuvants are available. However, vaccination is prohibited in many countries, because a differentiation between vaccinated and infected animals (DIVA) by using such vaccines is not possible.

This problem has been overcome by the generation of recombinant viruses, e.g. Newcastle disease viruses which express the hemagglutinin protein of HPAIV. Vaccination with these viruses confers protection against NDV and AIV and allows easy serological differentiation between AIV infected and vaccinated animals.

Here, different NDV recombinants with H5 or/and N1 genes inserted at different sites of the NDV genome were used for immunization of three week old chickens which were challenged three weeks after immunization with three different HPAIV of H5 subtype.

Material and methods

Recombinant NDVs which express H5 alone or in combination with N1 of HPAIV Vietnam H5N1 were generated using lentogenic NDV Clone 30 as backbone. For the insertion of foreign genes at different locations single restriction sites were inserted by site directed mutagenesis. Transfections were done as described previously (1). Recombinant viruses were propagated on embryonated chicken eggs and characterized by replication kinetics, Western blot analyses, indirect immunofluorescence of infected cells and determination of the intracerebral pathogenicity index (ICPI). To evaluate protection against three different HPAIV of subtype H5, three groups of three week old specific pathogen free chickens were ocolunasally immunized with 10^6 mean egg infectious doses (EID50) of one out of three generated recombinant NDVH5. Challenge infections of immunized and non-immunized control chickens were performed oculonasally with three different HPAIV of H5 subtype three weeks after immunization. Chickens were observed daily for signs of disease and a clinical score was calculated which represents the mean value of all chickens per group for indicated periods. Blood samples were collected before immunization, at day 10 and 20 post immunization and at the end of the experiment. Furthermore, oropharyngeal and cloacal swabs were collected at different days after challenge infection. AIV RNA was detected by real-time RT-PCR based on amplification of the M gene as described (2).

Results

Both H5 and N1 open reading frame of HPAIV H5N1 (Vietnam) were introduced as additional transcription units flanked by NDV specific gene start and gene end sequences as well as non-coding regions derived from NDV HN gene resulting in the isolation of two recombinant NDV which express the HPAIV H5 protein and one NDV which express both H5 and N1. All recombinants replicated well in embryonated chicken eggs and in chicken embryo fibroblasts. NDV proteins as well as AIV H5 or N1 proteins were simultaneously detected in infected cells by In indirect immunofluorescence. Western blot analyses demonstrated the presence of AIV H5 or/and N1 in lysates of cells infected with the respective AIV protein expressing recombinant NDV. Virulence of recombinant NDV was evaluated by determination of the ICPI value, which was zero for all of them, confirming the suitability of these viruses as attenuated live vaccine. The protective efficacy of a single immunization was evaluated in chickens. All animals remained healthy after immunization. The non-immunized chickens developed severe disease and died after challenge infection. Challenge infections of immunized chickens were carried out with homologous HPAIV or two different heterologous HPAIV of the same hemagglutinin subtype. Whereas, dependent on the vaccine virus, few chickens exhibited clinical signs with one fatal case after infection with HPAIV H5N1 Germany, they were fully protected after challenge infection with homologous H5N1 (Vietnam) or heterologous HPAIV H5N2. Shedding of challenge virus by immunized animals was strongly reduced as analyzed by detection of viral RNA in swab samples and restricted to only a few of the immunized birds. Detection of antibodies against AIV proteins other than those expressed by recombinant NDV would be indicative for challenge virus replication. Sera of immunized chickens were tested in an AIV NP-ELISA. Whereas AIV NP-specific antibodies were absent before challenge infection, several of the immunized birds seroconverted within 21 days after challenge infection.

Discussion and conclusions

Recombinant NDV which express immunogenic proteins of HPAIV from different position within the NDV genome are suitable as vaccine viruses. The results of the animal experiment suggest that a close match between the influenza hemagglutinin in vector vaccines and field virus could play a role in protection against HPAIV. Recombinant NDV-AIV which can be easily adapted to the current situation and administered by mass application has the potential to become important for control of two most devastating poultry diseases.

Acknowledgement

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DEVELOPMENT OF FOWL ADENOVIRUS 9 BASED VECTOR VACCINE EXPRESSING THE HEMAGGLUTININ GENE OF AN H5N1 INFLUENZA VIRUS

Dan-Hui YANG[1], Juan C. CORREDOR[1], Peter J. KRELL[2], Éva NAGY[1]

[1] *University of Guelph, Department of Pathobiology, Ontario Veterinary College, Guelph, Canada;*
[2] *University of Guelph, Department of Molecular and Cellular Biology, Guelph, Canada;*

Keywords: fowl adenovirus, influenza haemagglutinin, vaccine expression vector

Introduction and objectives

Fowl adenoviruses (FAdVs) are non-enveloped viruses with icosahedral symmetry and about 70-90 nm in diameter within the family *Adenoviridae*, genus *Aviadenovirus*. FAdV dsDNA genomes are about 10 kb longer than the 30-36 kb of the mastadenoviruses. Fowl adenoviruses have a worldwide distribution and appear to be ubiquitous on poultry farms. Certain FAdVs are more pathogenic than others, and diseases, such as inclusion body hepatitis, are more often associated with isolates belonging to serotypes 2, 4, 6, 8 and 11. Low pathogenic FAdVs are good candidates as vector viruses for use in poultry. We already developed a Fadmid based platform for construction of recombinant viruses based on fowl adenovirus 9 (FAdV-9) (Ojkic and Nagy, 2000; 2001). In our earlier work we showed that the tandem repeat region 2 (TR-2) which is on the right end of the genome can be deleted and replaced with a foreign gene (Ojkic and Nagy, 2001). We also demonstrated that chickens can be immunized with the recombinant virus and the virus could be administered through the drinking water and feed (Ojkic and Nagy, 2003). From these results we felt that FAdV-9 could be used as a vaccine against poultry diseases. In this study we wished to identify additional non essential regions for insertion and expression of a foreign gene. We chose the influenza virus haemagglutinin gene inserted into this non essential region as a model to evaluate the feasibility and efficacy of this vaccine vector.

Materials and methods

To identify additional sites for insertion we conducted an extensive deletion analysis of the left end of the genome of FAdV-9 (Corredor and Nagy, 2009). Our FAdmid technology was used for these studies to generate a series of deletions of an infectious FAdV-9 plasmid clone in a bacterial background. To determine if the mutated (deletions) FAdmids were viable (infectious) they were isolated from bacteria and tested for virus production following transfection of CH-SAH cells. As a model to test our system, we generated, by homologous recombination with the FAdmid in bacteria, two recombinant viruses, FAdV-9ΔL-HA-R and FAdV-9ΔL-HA-L, in which an influenza virus A HA coding sequence in rightward (R) and leftward (L) orientations replaced one of the larger non essential regions. Recombinant adenoviruses were recovered subsequent to transfection of chicken hepatoma (CH-SAH) cells. Expression of the HA protein was monitored by Western immunoblotting using anti-avian influenza A (H5N1) HA polyclonal antibody. The activity of the recombinant HA protein was also examined by hemadsorption and hemagglutination assays. Growth efficiency, plaque morphology and CPE of HA-R and HA-L FadVs and wt FAdV-9 were examined and compared.

Results

Based on the deletion analysis, we identified a 2.4 kb non-essential region in the left end of the FAdV-9 genome (Corredor and Nagy, 2009). We reasoned that we could clone a foreign vaccine gene into this non essential region as described above. Here we report on the generation and characterization of two recFAdV-9 viruses expressing the full-length hemagglutinin (HA) gene of an avian influenza virus A, inserted in two orientations in the left end region. We were able to generate both FAdV-9ΔL-HA-R and FAdV-9ΔL-HA-L using our FAdmid technology. Proper insertion in the deleted non essential left region was confirmed by PCR and sequencing. Expression of HA was detected by Western blotting for both L and R orientations. However, higher levels of HA expression were detected for HA cloned in the rightward orientation. Expression of HA on the surface of FAdV-9ΔL-HA-R infected cells was demonstrated by heamadsorption with chicken RBCs. We could not detect any difference in CPE, plaque morphology and growth kinetics between the recombinant viruses and FAdV-9. The recombinant viruses were stable over four passages.

Discussion and conclusions

We successfully demonstrated that a 2.4 kb region of the left end of the FAdV-9 genome could be removed and replaced with a foreign gene and still generate a viable virus. We also demonstrated, using the HA gene as a model, that a foreign gene encoding a protein important in vaccination, could be cloned and expressed in this left end region without compromising virus replication in cell culture. Moreover we demonstrated that the gene cloned into this region was expressed as a biologically functional protein. Thus we have established a suitable platform for expressing any foreign gene for an antigen in an FAdV-9 vaccine vector backbone.

Acknowledgements

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IMMUNOGENICITY OF FOWLPOX VECTORED AND INACTIVATED AVIAN INFLUENZA VACCINES IN CHICKENS WITH MATERNAL ANTIBODIES

Alexandra RICHARD-MAZET[1], Mieke STEENSELS[2, 3], Sandrine CHANAVAT-BIZZINI[1], François-Xavier LE GROS[1], Michelle DUBOEUF[1], Bénédicte LAMBRECHT[2], Thierry VAN DEN BERG [2], Michel BUBLOT[1]

[1]Merial S.A.S., R&D, 69007 Lyon, France;
[2]Veterinary and Agrochemical Research Centre (VAR), 1180 Brussels, Belgium;
[3]Fac. of Biosc. Engin., Dpt of Biosystems, Division of Gene Technol., KULeuven 3001 Heverlee, Belgium

Keywords: avian influenza, chickens, fowlpox, maternal antibodies, vector, vaccine

Introduction and objectives

Fowlpox (FP) vectored avian influenza (FP-AI) vaccines (1, 2) are used in one-day-old broilers in Central America in low pathogenic avian influenza (LPAI) H5N2 endemic areas. The FP-AI (vFP89) commercial vaccine was shown to be protective in field broilers with anti-FP and/or anti-AI maternally-derived antibodies (MDA) and challenged with either a HPAI or a LPAI Mexican H5N2 challenge. It was also shown to protect SPF chickens against H5N1 isolates (3). The objective of studies presented here was to further analyze the effect of MDA on FP-AI vaccine immunogenicity.

Materials and methods

Vaccines: The fowlpox recombinant virus vFP89 is the active ingredient of the TROVAC®-AIV H5 vaccine (Merial Select, Inc., Gainesville, GA; TROVAC is a registered trademark of Merial in the USA and elsewhere) that expresses the native HA gene from the HPAI A/turkey/Ireland/1378/1983 (H5N8) isolate (1, 2). The fowlpox recombinant virus vFP2211 contains a synthetic version of the HA gene modified at the cleavage site of the HPAI A/chicken/Indonesia/7/2003 H5N1 isolate in the same fowlpox (TROVAC) vector as vFP89 (2, 4). The experimental H5N9 inactivated vaccine (inH5N9) contained the A/chicken/Italy/22A/1998 (H5N9).

Study 1 : One-day-old chicks hatched from SPF hens vaccinated twice with inH5N9 (wk3 & 17) and once with fowlpox (wk9) (MDA-H5N9+FP) were immunized with either vFP2211 (5 $\log_{10}$ $TCID_{50}$/dose) at D0 and with 0.5ml of inH5N9 at D21 (prime-boost) or with 0.5ml of inH5N9 only at D21. HI tests were performed with H5N9 and H5N1 (NIBRG14 containing the HA from clade 1 H5N1 A/Vietnam/1194/2004) antigens.

Study 2 : One-day-old SPF or MDA-H5N9+FP chicks were immunized with either vFP89 (3 $\log_{10}$ $TCID_{50}$/dose) at D0 and inH5N9 (0.5ml) at D21 (prime-boost) or at both D0 (0.2ml) and D21 (0.5ml) with inH5N9. HI tests were performed with H5N9, H5N8 and H5N1 (NIBRG14) antigens.

Study 3 : One-day-old chicks hatched from SPF hens vaccinated with once with vFP89 (wk0), twice with inH5N9 (wk17 & 63) and twice with fowlpox (wk9 & 63) (MDA-H5N8/H5N9+FP) were immunized with either vFP89 or vFP2211 (3 $\log_{10}$ $TCID_{5}$0/dose) at D0 and inH5N9 (0.5ml) at D21, or with inH5N9 at both D0 (0.2ml) and D21 (0.5ml). Birds were challenged at D31 with 10^7 EID_{50} A/crested_eagle/Belgium/01/2004 (H5N1 clade 1).

Study 4 : One-day-old SPF or chicks hatched from SPF hens vaccinated either 3 times (wk3, 6 & 16) with inH5N9 (0.3ml) (MDA-H5N9), or twice with fowlpox (wk 4 & 12) (MDA-FP), or with vFP89 (wk0), fowlpox (wk12) and inH5N9 (wk16) (MDA-H5N8/H5N9+FP) were immunized with either vFP89 (3 $\log_{10}$ $TCID_{50}$/dose) at D0 and inH5N9 (0.3ml) at D14 or at D21 only with inH5N9 (0.3ml). HI tests were performed with H5N9, H5N8 and H5N1 (NIBRG23 containing the HA from clade 2.2 H5N1 A/turkey/Turkey/1/2005) antigens.

Results

Study 1: The priming effect of vFP2211 in MDA-FP+H5N9 chicks was clearly detected by HI test at D42 and D84 against both H5N9 and H5N1 antigens when compared to one administration of inH5N9 (see Fig. 1).

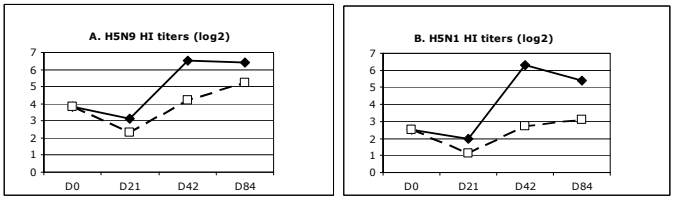

Figure 1: H5N9 (A) and H5N1 (B) HI titers induced by a vFP2211 at D0/inH5N9 at D21 prime-boost scheme (plain line-black diamond) or inH5N9 (dotted line-open square) at D21 in MDA-FP+H5N9 chicks.

Study 2: The priming effect of vFP89 in MDA-FP+H5N9 chicks was clearly detected by HI test at D42 against the three H5 tested antigens when compared to two administrations of inH5N9. However, the prime-boost regimen induced 1-2 log2 lower HI titers in MDA-FP+H5N9 compared to SPF chicks (see Fig. 2).

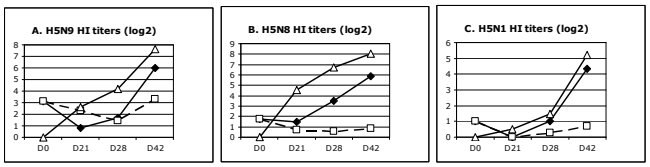

Figure 2: H5N9 (A), H5N8 (B) and H5N1 (C) HI titers induced by a vFP89 at D0/inH5N9 at D21 prime-boost scheme (plain line-black diamond) or 2 inH5N9 at D0&D21 (dotted line-open square) in MDA-FP+H5N9 chicks or prime-boost in SPF birds (plain line-open triangles).

Study 3: No HI detectable HI titers were induced by the prime-boosts or the two administrations of inH5N9 in MDA-H5N8/H5N9+FP chicks hatched from hyperimmunized hens. Furthermore, most vaccinated chickens died after challenge (11/11, 11/12 & 10/11 for the prime-boost with vFP89 or vFP2211 and the 2 inH5N9 administrations, respectively).

Study 4: The highest MDA interference was observed with one inH5N9 administration at D14 in MDA-H5N9 chicks compared to SPF (3-4 log2 difference; see Fig 3). Interference in MDA-H5N8/H5N9+FP was lower (1-2 log2 difference; not shown). The vFP89 priming could clearly overcome the interference of H5N9 MDA on inH5N9 vaccine take (see Fig.3). MDA interference on the immunogenicity of the prime-boost regimen was the highest (~2log2 at D42) in chicks hatched from breeders vaccinated with the prime-boost regimen (MDA-H5N8/H5N9+FP). In MDA-FP chicks, vFP89-induced H5N8 HI titers at D14 were similar to those in SPF chicks; however, after the inH5N9 boost, D42 HI titers were slightly lower than in SPF (1-2 log2 difference; not shown).

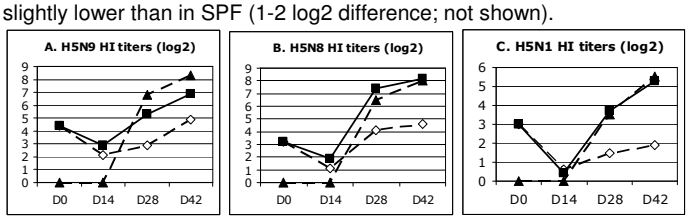

Figure 3: H5N9 (A), H5N8 (B) and H5N1 (C) HI titers induced by one inH5N9 administration at D14 in SPF (dotted line-black triangle) or in MDA-H5N9 (dotted line-open diamond) or by vFP89 at D0/inH5N9 at D14 prime-boost scheme (plain line, black square) in MDA-H5N9 chicks.

Discussion and conclusions

The immunogenicity of inH5N9 vaccine was severely impaired in chicks hatched from inH5N9-vaccinated breeders. This MDA interference could be overcome by priming the chicks at day-of-age with a FP-AI vector. Furthermore, this prime-boost scheme induced a broader cross-reactivity against different H5 antigens (4). Similarly, the highest interference observed on the prime-boost was observed in chicks hatched from breeders vaccinated with the same prime-boost scheme. In chicks hatched from hyperimmunized breeders, neither the prime-boost nor 2 administrations of inH5N9 induced HI titers or H5N1 protection. Thus, the immunogenicity of vaccines in young chicks with MDA depends on the vaccination scheme and the type of vaccine used in their breeders. Additional vaccination/challenge studies need to be done in field conditions to find the optimal vaccination schemes to be used in breeders and in the progeny with minimal MDA interference. In our experimental conditions and in the field in Mexico (5), the prime-boost scheme has shown to be efficient to induce immunity in young chicks with FP and AI MDA.

Acknowledgements

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EFFICACY OF C-STRAIN VACCINE IN DOMESTIC PIGS CHALLENGED WITH GENOTYPE 2.1 AND 3.3 CLASSICAL SWINE FEVER VIRUS SHORTLY AFTER VACCINATION

Helen E. EVERETT[1], Simon P. GRAHAM[1], Derek CLIFFORD[2], Helen JOHNS[1], Felicity J. HAINES[1], Olubukola SOSAN[1], F. Javier SALGUERO[3], Trevor W. DREW[1], Helen R. CROOKE[1]

Veterinary Laboratories Agency - Weybridge, Department of Virology[1]; Animal Service Unit[2]; Pathology[3]; New Haw, Surrey, UK

Keywords: classical swine fever virus, emergency vaccination, vaccine efficacy,

Introduction and objectives

Classical swine fever virus causes a haemorrhagic disease of pigs that has profound social and economic consequences. Emergency vaccination to assist in control of classical swine fever outbreaks is not banned under EU legislation, but since 1990 most control strategies have been based on stamping out. Recently, large-scale culling to control animal diseases has been questioned and the use of some form of vaccination to control future outbreaks is being increasingly discussed. Emergency vaccination can either be suppressive, where animals are vaccinated and subsequently culled, or protective, where animals are allowed to live out their normal life. Current modified live vaccines are safe and effective but due to the inability to identify infection in vaccinated animals by serology, these vaccines are likely to be limited to suppressive strategies in large-scale domestic pig production. For a suppressive strategy the vaccine must provide rapid protection against virus transmission for all strains of virus that may be encountered. It is well known that C-strain vaccines provide protection from clinical signs within a week of vaccination[1,2] However, no studies have quantified the efficacy in preventing infection of unvaccinated, in-contact animals at very early time points. In an emergency situation it is important to have some prior knowledge on the likely efficacy of a vaccine against the strain in question. C-strain vaccines are effective against a broad range of strains[1]. However, genotype 3.3 strains, which have not, thus far, been isolated outside of the Far East, have not been extensively studied. We recently characterised a genotype 3.3 virus, CBR/93, which is also antigenically diverse as it reacts only poorly with a monoclonal antibody that recognises all other CSFV previously tested[3].

We therefore investigated how rapidly vaccination with C-strain vaccine (Riemser Arzneimittel AG) protects against infection of in-contact animals after challenge of pen-mates with a genotype 2.1 strain, that caused a recent outbreak in the UK (UK2000/7.1), and the genotype 3.3, CBR/93 strain.

In previous experiments we detected virus-specific production of IFN-γ from peripheral blood leukocytes (PBL) isolated from C-strain vaccinated, but not UK20007.1 infected, pigs[4]. To investigate the association that this cytokine may have to vaccine induced protection, which may aid the development of future generation of CSFV vaccines, we explored the kinetics of virus-specific IFN-γ responses from PBL, isolated over the course of the experiments.

Materials and methods

Groups of six, 10-week old pigs were vaccinated i.m. 5, 3 or 1 day/s prior to i.n. challenge with either UK2000/7.1 or CBR/93. Groups of 4 unvaccinated pigs were included as controls. In all cases the challenged animals were re-introduced to unvaccinated, in-contact animals 24 hours post challenge. Clinical scores and biochip temperatures were monitored twice daily. Blood and nasal swabs were taken at 2-3 daily intervals and the number of copies of viral RNA determined by real time RT-PCR. Platelet and white blood cell counts were monitored by flow cytometry. Macro and histopathological lesions were compared using a pathological scoring system[3]. Antibody responses were measured using an E2 ELISA. Virus-specific T-cell IFN-γ responses were monitored by stimulating isolated PBL with challenge and vaccine virus *in vitro* and detecting IFN-γ in culture supernatants with a swine IFNγ ELISA.

Results

Unvaccinated animals inoculated with either strain developed clinical signs, leukopenia, viraemia, had high levels of viral RNA in nasal swabs and transmitted the virus to in-contact animals within 14 days of inoculation. Animals vaccinated 5 days prior to challenge with either strain were completely protected, no viral RNA was detected in nasal swabs, animals did not develop leukopenia or pathological lesions and in-contact animals did not develop disease. Animals challenged with CBR/93 three days after vaccination were protected clinically, although low levels of RNA were detected in the blood of some challenged animals the in-contact animals did not become infected. In contrast, challenge with UK2000/7.1 three days after vaccination did not prevent infection of in-contact animals, although 5 of the 6 UK2000/7.1 challenged animals had no, or only few, clinical signs and lower levels of leukopenia, viraemia and viral RNA in blood and nasal swabs compared to the unvaccinated controls. Challenge with either strain 1 day after vaccination, did not protect the in-contact animals from infection. However, with both challenge strains around half of the animals vaccinated at day -1 were protected to some extent from the virus, with fewer clinical signs, less leukopenia and viraemia and, importantly, were shedding less virus in nasal swabs.

High levels of antibody against E2 were detected in animals at around 12 days post challenge. Notably, animals that were solidly protected against challenge with either strain had PBL that produced high levels of virus-specific IFN-γ by 6 days post-challenge. Most animals that were partially protected also had IFNγ secreting PBL, although the levels were lower and delayed. In comparison animals in the unvaccinated groups, and those that were not protected by vaccination, had no IFN-γ responses.

Discussion and conclusions

These data indicate that use of this C-strain vaccine provides solid protection against transmission of diverse CSFV strains by 5 days post vaccination. Vaccination would also provide benefit in the event of exposure to virus at earlier time points by reducing the subsequent viral load shed into the environment and hence reducing the potential for further virus spread. This benefit should be considered alongside the fact that there was a reduction in clinical signs in vaccinated and subsequently infected animals. This may mask the early identification of infection in vaccinated herds, although the presence of challenge virus in the blood of vaccinated animals could be identified by RT-PCR. Any emergency vaccination strategy would still have to be accompanied by carefully considered monitoring and biosecurity measures.

The data also support the hypothesis that the presence of high levels of IFNγ secreting T-cells provide a marker of cell-mediated mechanisms that are able to control CSFV, and that understanding what is required to stimulate these mechanims will benefit future marker vaccine development.

Acknowledgements

We are grateful to Dr S. Parchariyanon of NIAH Thailand, the EU commission & Riemser Arzneimittel for donating the virus and vaccine used in this study. We also thank the numerous members of the virology, pathology and animal services departments of the Veterinary Laboratories Agency who assisted with different aspects of this project.

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EVALUATION OF A NEW VACCINATION STRATEGY AGAINST BLUETONGUE BASED ON EXPRESSING A GROUP SPECIFIC ANTIGEN (VP7)

Coraline BOUET-CARARO[1], Vanessa CONTRERAS[2], Annie FOURNIER[1], Marion SZELECHOWSKI[1], Emmanuel BREARD[1], Pierre RUSSO[3], Richard THIERY[3], Isabelle SCHWARTZ-CORNIL[2], Stéphan ZIENTARA[1], Bernard KLONJKOWSKI[1]

[1]AFSSA, INRA, ENVA, UMR 1161 Virologie, Ecole Nationale Vétérinaire d'Alfort, Maisons-Alfort, France;
[2]INRA, UR 892 Virologie et Immunologie Moléculaires INRA, Jouy en Josas, France;
[3]AFSSA, Unité Pathologie des Petits Ruminants, Sophia Antipolis, France

Keywords: adenovirus, bluetongue, vaccine

Introduction and objectives

Bluetongue virus (BTV), an orbivirus of the Reoviridae family, is the cause of a haemorrhagic disease of ruminants, mainly sheep, occasionally cattle and wild ruminants. The BTV is a non-contagious virus transmitted from one animal to another by the bite of insects of the Culicoides genus. Up to recent years, BTV was known to be endemic in tropical and sub tropical regions but the recent emergence of various serotypes (1, 2, 4, 6, 8, 9, 11 and 16) in Europe has led to dramatic economic losses. Bluetongue is an Office International des Epizooties (OIE) listed disease because of its large economic impact resulting from loss of productivity, mortality, restrictions on animal movements and the costs of setting control measures. Attenuated or inactivated vaccines are currently available but the protection they afford is serotype specific and they do not allow easy differentiation between vaccinated and infected animals (no DIVA vaccines). Thus, other vaccination strategies, such as virus like particles and recombinant vectors, should be developed to generate safe and cross protective DIVA vaccines [2].

To this end we have constructed a recombinant canine adenovirus (CAV) containing cDNA copie of segment 7 of BTV serotype 2 (BTV2 strain Corsica 2000). The construction of this recombinant adenovirus and its evaluation in sheep will be presented.

Material and methods

Construction of non replicative adenovirus vector: we generated a CAV2 vector with an intact E3 region and a VP7 expression cassette set in the E1 gene sequence leading to the inactivation of this gene, thus to the replication-deficient vector CAV2-VP7 R^0. The VP7 gene expression is under the control of the human cytomegalovirus (CMV) early enhancer/promoter and the expression cassette has been inserted in the reverse orientation to the CAV2 genome sequence.

In vitro expression of the VP7 gene: expression of VP7 has been assessed by immunofluorescence assay on Dog kidney cells (DK) and Vero cells.

Sheep inoculation: humoral immune response and protection afford by CAV2-VP7 R^0 were evaluated in a first animal experiment. Twenty-one Pré-Alpes crossbreed sheep, supplied from the Experimental Station of Sophia-Antipolis (AFSSA), were randomly divided into 5 groups. In a second set of experiments eight Préalpes female sheep, supplied from the Experimental breeding of Brouessy (INRA), were randomly assigned to two groups of 4 sheep to evaluate the specific anti-VP7 cell-mediated immune response. First group was inoculated with CAV2-VP7 R^0 and second group with CAV2-N R^0 (replication-deficient vector negative control with an irrelevant antigen).

Serological assays for CAV2-specific antibodies and for VP7-specific antibodies were performed, as well as nodes collection and evaluation of the cellular immune response.

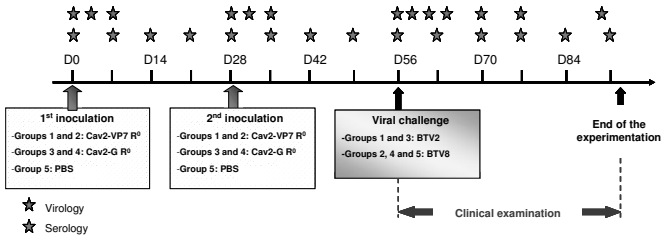

Figure 1. Protocol for evaluation of CAV2-VP7 R^0 vaccine in sheep.

Results

CAV2-VP7 R^0 genome was obtained by different cloning steps and homologous recombination. The recombinant virus was produced in DK cells transcomplemented with the E1 region of CAV2. Our vector CAV2-VP7 R^0 supports *in vitro* expression of VP7 gene. CAV2-VP7 R^0 elicits antibodies directed against VP7 in sheep, elicits specific T-cell responses and diminishes somewhat viral burden after homologous challenge.

Discussion and conclusions

Immunodominant serotype cross reactive T-cell determinants are located within the NS1 non structural protein and the structural proteins of BTV cores, namely VP7, VP3 (major core proteins) and VP1, VP4 and VP6 (minor proteins). VP7 vectored by a recombinant capripox virus has proved to be effective in protecting sheep against a virulent heterologous BTV challenge [3]. For this reason, we have constructed a replication defective canine adenovirus serotype 2 that express the VP7 of BTV2 (CAV2 -VP7 R^0). The purpose of this study was to investigate the protection as well as the cross protection afford by this recombinant vaccine in sheep. Our data shows that although this recombinant viral vaccine was effective in inducing humoral and cellular responses against the VP7 protein and was effective in reducing the viral load but it did not afford clinical protection against either homologous (BTV2) or heterologous (BTV8) viral challenges. The differences shown between BTV2 and BTV8 challenges could arised from the differences inherent to the doses and strength of the two strains used in this trial. But in both cases anti-VP7 antibodies and anti-VP7 specific T-cell responses were not sufficient to protect against bluetongue challenge.

These results are in accordance with those recently obtained by collaborators with poxvirus vectors [1] but are in contrast to those obtained by Wade-evans *et al.* [3]. Preliminary results obtained in sheep with a model antigen vectorised by Cav2 allow us to develop other candidate vaccines in particular expressing VP2 protein of BTV2.

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EVALUATION OF THE RESPONSE INDUCED BY VLP VACCINES FOR BLUETONGUE VIRUS SEROTYPES 1 AND 1&4 IN SHEEP

Ana C. PÉREZ DE DIEGO[1], Belén RODRÍGUEZ[1], Rob NOAD[2], T.N. ATHMARAN[2], Meredith STEWART[2], José M. SÁNCHEZ-VIZCAÍNO[1], Polly ROY[2]

[1]*Centro de Vigilancia Sanitaria Veterinaria, VISAVET, Animal Health Department, Faculty of Veterniary Science, Universidad Complutense de Madrid, Spain;* [2]*London School of Hygiene & Tropical Medicine, London, UK*

Keywords: bluetongue virus, VLP vaccine

Introduction and objectives

Vaccination is a basic measure to control Bluetongue Disease *(Savini et al., 2008)* as it has been demonstrated recently in Spain after the eradication of BTV serotype 4.

The novel Virus Like Particles (VLP) vaccines present two fundamental advantages: the possibility to create polyvalent vaccines with different VP2 proteins from different serotypes and the capability to distinguish between infected and vaccinated animals, since vaccinated animals will not present antibodies for non structural proteins, due to the fact that the vaccine only contains 4 structural proteins. *(Noad and Roy, 2003)*

The aim of this study was to evaluate the response to a monovalent VLP vaccine for BTV serotype 1 and a polyvalent VLP vaccine for BTV serotypes 1&4, by the clinical survey, ELISA test to detect antibodies, and RT-PCR to detect the viraemia.

Materials and methods

The vaccines used were developed by VLP technology, and were a monovalent vaccine for serotype 1 and a bivalent vaccine for serotypes 1 & 4. The adjuvant administered instead of the vaccine, in the control animals, was the Montanide ISA 296 VG which is the one used also in the VLP vaccines. The animals for this study were 26 Merino sheep, male and female, about 7-8 month old at the beginning of the experiment. They were separated in 5 groups. Group A, 8 animals vaccinated with VLP for BTV serotype 1 (BTV1) and challenged with the same serotype. Group B, 6 animals vaccinated with VLP for BTV1&4 and challenged with BTV1. Group C, 3 animals that were not vaccinated and they were challenged with BTV1. Group D, 6 animals vaccinated with VLP for BTV 1&4 and challenged with BTV4. Finally Group E: 3 animals that were not vaccinated but challenged with BTV 4. Following a previously established calendar the vaccination was carried out on day 0 of the experiment and the revaccination on day 20, the challenge was performed on day 48 and the trial ended with the euthanasia of the animals on day 75. Groups A, B and C were challenged with BTV1 and the groups D and E with BTV4. Following the calendar the temperature was measured with rectal thermometer and with infrared thermography, and sera and blood samples were collected. To carry out the clinical exploration, we based on previous experiments (Perrin et to., 2007) from which, a table was developed with the main clinical signs of BTV disease, assigning different scores to each of them, according to its importance. Sera samples were analyzed by a competitive ELISA test for the detection of antibodies against VP7 protein (POURQUIER Bluetongue Competitive ELISA). Blood was analyzed by RT-PCR, by the use of primers and a specific probe following the protocol established by Toussaint et al. (2007). Between days 69 and 71 the sheep from gropus C and E were sacrificed and the necropsy of these animals and 2 animals from each vaccinated groups (A, B and D) was performed. The clinical trial was completed at day 75, when the remaining animals were sacrificed.

Results

The information of clinical exploration recorded from the groups A, B and D indicates that the vaccinated animals do not present the clinical disease. The animals of the groups C and E showed symptomatology, and it was more pronounced in the animals of the group C. In both groups the clinical symptomatology appeared about days 8 and 11 post infection (pi) and it lasted in some cases until day 19 pi. The rectal temperature average of the groups C and E suffered an increase since day 8 after the challenge. For infrared thermography temperatures, the results obtained from one of the animals from group C, indicated that the temperature registered in the eyes, was 38,4 ºC and remained the same until the challenge. However, it was raised up to the 40,1 ºC on day 5 pi, coinciding with the moment of the maximum rectal temperature (41,9 ºC). At the necropsy, the pathological signs observed in the Group C were: predominance of white splenic zones (2/3), reactive bone marrow (1/3), generalized lymphnodes hypertrophy (3/3) and alveolar oedema (2/3). Group E presented predominance of white splenic zones (1/3), and generalized lymphnodes hypertrophy (2/3) .In groups A and B the animals presented positive results to the ELISA test from day 15 after the revaccination, with the exception of an individual (1/8), from wich antibodies were not detected until day 10 pi. In the group C antibodies were detected from day 10 pi. The average of the animals of the group D shows the presence of antibodies since day 15 after the revaccination and since day 20 pi in group E. In the group A only six animals (6/8) show positive results to the RT-PCR, on day 6 pi. All the animals of the group B show negative results except on day 6 pi. The control group for serotype 1 (Group C) shows positive results since day 3 until day 20 pi. In 3 of the animals from group D (3/6) positive results were obtained on day 6 pi. 2 animals of this group (2/6) were also positive 13 days pi. In the Group E positive results were detected from day 5 up to day 20 pi.

Discussion and conclusions

The use of a score to evaluate the symptoms appears to be a suitable form to compare the results among different studies. The animals from the control groups showed variable BTV specific symptomatology. The observed symptomatology was more severe in the animals infected with BTV 1 that in those infected with BTV 4, which coincides with field observations in Spain. The high temperature in the non-vaccinated animals and its marked increase from day 8 post-inoculation were expected. The preliminary information from infrared thermography, indicates that this technique is a rapid and effective tool that reduces the level of stress in the animals, and it will be suitable to detect the changes of temperature derived from feverish processes specially registering the temperature from the eyes. The pathologic findings at the necropsy are not obvious; it is probably due to the fact that the time chosen for the assessment of the necropsies was not the most appropriate since the animals were in the phase of recovery from the disease. The absence of clinical symptomatology, as well as of fever and of pathologic signs compatible with Bluetongue in the vaccinated animals, indicates the capacity of the VLP1 and VLP 1&4 vaccines to avoid the appearance of the clinical signs. In some vaccinated animals the presence of antibodies against VP7 were detected from day 10 post vaccination, in others from day 20 post vaccination and the major result of antibodies was obtained on day 35 post vaccination. Previous studies developed with BTV 2 inactivated vaccine in which the presence of VP2 neutralizing antibodies was analyzied, yielded similar results. (Hamers et al. 2009) Thus our data demonstrates that that the temporary development of the immune response of the VLP vaccines is similar to that of the inactivated vaccines. The presence of antibodies in the vaccinated animals indicates the capacity of the VLP vaccines to stimulate the humoral response. RT-PCR's results indicate a clear difference between vaccinated animals, with a brief peak of viraemia that lasts only one day, and not vaccinated, whose viraemia is longer. The VLP vaccines proved to be effective to diminish the viraemia after the challenge with BTV.

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DETERMINATION OF THE MINIMUM PROTECTIVE DOSE FOR BLUETONGUE SEROTYPE 2, 4 AND 8 VACCINES IN SHEEP

Jacob MODUMO[1], Estelle H. VENTER[2]

[1]*Onderstepoort Biological Product Ltd., Private Bag X07, Onderstepoort, 0110, South Africa;*
[2]*Department of Veterinary Tropical Disease, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, 0110, South Africa*

Keywords: bluetongue virus, minimum dose, protection, vaccine

Introduction and objectives
Bluetongue (BT) is a non-contagious, insect-transmitted disease of certain domestic and wild ruminants that is caused by the Bluetongue virus (BTV). To date, 25 known serotypes of BTV have been described worldwide (Chaignat, 2009), 21 of these serotypes are known to occur in South Africa, where the disease is endemic and of which 15 are considered to be pathogenic for sheep (Gerdes, 2004). Since 1999 BT serotypes 2, 4, 6, 8, 9 and 16 have been prevalent in Europe Vellema, 2008; Batten *et al*., 2008). The BT live attenuated virus vaccine has been used successfully in the control of BT in southern Africa and Europe. This vaccine is released by the Onderstepoort Biological Products at a titre of 5×10^4 PFU/ml (Dungu *et al*., 2004). However, the safety of the vaccine used at this titre, as well as the possible development of viraemia and clinical signs post vaccination (p.v.) raised concerns and prompted the need to investigate the possibility of reducing the current BT vaccine titre to below 10^4 PFU/ml without decreasing its ability to protect.

Materials and methods
A total of 83 merino sheep were used and divided into 2 groups, Group A and B. Three BTV serotypes, 2, 4 and 8 monovalent vaccines each with the following titres: 10^2, 10^3 and 10^4 PFU/ml were produced and injected into both groups of sheep; 12 sheep per serotype and 4 sheep per titre were used. Positive and negative controls for each serotype were used. Blood samples were taken from animals on days 0, 3, 6, 9, 12, 15, 18, 21, 25 and 28 p.v. and tested for viraemia. Seroconversion was tested p.v. on days 0, 3, 9, 15, 21, 6 weeks (Group A and B), 3 and 4 months (Group B). Each group was then challenged, Group A was challenged at 6 weeks p.v. using BTV infected blood and Group B challenged at 4 months p.v. using cell cultured material. Sheep were evaluated for 14 days post challenge (p.c.) using a clinical reaction index and viraemia was tested for 21 days p.c.

Results
In both groups A and B sheep, no local or systemic reactions were seen in any of the serotypes p.v. and only transient fever was seen within the first 14 days p.v. which generally lasted only one to two days. Seroconversion was demonstrated in all sheep vaccinated with all titres of BTV2 and 8, but only 50% of sheep reacted with low titre of 10^2 PFU/ml of BTV4.

Sheep challenged with serotype 4 developed mild clinical signs for less than 3 days. Sheep challenged with cell cultured BTV serotype 2 also showed mild clinical signs and also developed viraemia that lasted for less than 3 days which was also demonstrated in sheep vaccinated with a titre of 10^4 PFU/ml. Sheep vaccinated with serotype 8 did not demonstrate any form of viraemia both p.v. and p.c. at all titres.

Discussion and conclusions
It was clearly shown that BTV monovalent vaccine containing serotypes 2, 4 and 8 with titres below 10^4 PFU/ml can protect more than 90% of vaccinated animals against clinical disease. Although BTV 2 and 4 failed to protect against infection, all serotypes protected against the development of clinical disease when challenged with both BTV-infected blood and cell cultured material.

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EVALUATION OF VACCINES AGAINST BLUETONGUE VIRUS IN A MURINE MODEL

Javier ORTEGO[1], Noemí SEVILLA[1], Teresa RODRÍGUEZ-CALVO[1], Juan ANGUITA[2], Eva CALVO-PINILLA[1]

[1]Centro de Investigación en Sanidad Animal, CISA-INIA, Madrid, Spain;
[2]Department of Veterinary and Animal Sciences, University of Massachusetts Amherst, Amherst, USA

Keywords: bluetongue virus, IFNAR $^{(-/-)}$ mice

Introduction and objectives
Bluetongue (BT) is a non-contagious, insect-transmitted disease of domestic and wild ruminants caused by bluetongue virus (BTV). BT is a reportable disease of considerable socioeconomic concern and of major importance in the international trade of animals and animal products. Studies involving the natural host of BTV are limited by the complexity of the system, the scarce knowledge of the immune system of the natural hosts, and the need to have an animal facility with biosafety level 3. A laboratory animal model would greatly facilitate the studies of pathogenesis, immune response and vaccination against BTV. Herein, our objective was establishing a murine model susceptible to BTV and suitable for the evaluation of vaccination strategies against this virus.

Materials and methods.
Animal inoculation. LD50 values were calculated after intravenous inoculation of mice with 10-fold serial dilutions of BTV-4 (from 10^6 to 10^1 PFU/mouse). The amount of infectious virus from whole blood and homogenized tissues was measured by plaque assay on Vero cells.
Murine immunizations. A group of IFNAR $^{(-/-)}$ mice were immunized by two consecutive subcutaneous injections ZULVAC-BTV-4 ($1.5x10^6$ TCID50BTV-4) inactivated BTV-4 preparation (Fort Dodge Veterinaria, S.A.), administered 3 weeks apart. Sera were tested for BTV-4 neutralizing antibodies by Virus Neutralization Test (VNT). Mice were intravenously inoculated with 10^3 PFUs of BTV-4 (lethal dose) 3 weeks after the last immunization. Whole blood was collected in EDTA from all animals at regular intervals after inoculation and BTV challenge. The amount of infectious virus from whole blood was measured by plaque assay on Vero cells. Total RNA was extracted and a real-time RT-qPCR specific for BTV segment 5 was performed as described by Toussaint et al. (2007).

Results.
We have show that adult mice defficient in type I IFN receptor (IFNAR $^{(-/-)}$ mice) are highly susceptible to BTV-4 and BTV-8 infection when the virus is administered intravenously. Disease was characterized by ocular discharges and apathy, starting at 48 h post-infection and quickly leading to animal death within 60 h after inoculation. Infectious virus was recovered from the spleen, lung, thymus and lymph nodes. Furthermore, IFNAR $^{(-/-)}$ adult mice immunized with a BTV-4 inactivated vaccine showed the induction of neutralizing antibodies against BTV-4 (VNT 1.53±0.32) and complete protection against challenge with a lethal dose of this virus, while all nonimmunized animals died. Infectious virus was not detected in immunized mice but titers up to $3x10^4$ PFU/ml were observed at 5 days post-challenge in nonimmunized animals (figure 1). In addition, the presence of BTV genome in the blood of immunized and nonimmunized IFNAR $^{(-/-)}$ mice challenged with BTV-4 was analyzed by RT-qPCR. Nonimmunized mice were positive for BTV genome at days three or four after BTV infection (Ct: 27-29) and the presence of BTV genome was increased (Ct: 23-26) thereafter until animal death. In contrast, the majority of the immunized mice (n=5) were negative all the days post-challenge analyzed (Ct≥38). One of the vaccinated mice was positive (Ct: 30) at days four and five post-challenge and two of them at day 5 (Ct: 32) but in these three immunized mice the Ct was higher than in the non immunized and the presence of BTV-genome reverted to negative at day 7 post-challenge. Overall, these data indicate that protective immunity was achieved after vaccination.

Discussion and conclusions
BTV infects newborn mice (Brewer, 1998), but it will be necessary an adult animal model to allow studies of acquired immune response and vaccination against BTV. Genetically targeted mice lacking the β subunit of the IFN-α/β receptor (IFNAR$^{(-/-)}$ mice) are unable to establish an antiviral state and are highly susceptible to many viral infections, despite the presence of an otherwise intact immune system (Fiette et al, 1995). Bluetongue virus is a potent interferon alpha (IFN-α) inducer (Jameson et al, 1978). In addition, a temporal relationship of viremia and IFN-α activity has been observed in sheep infected with BTV, where IFN peak concentrations induced approximately a 90% decrease in virus titer (Foster et al, 1991). Here, we characterize a new animal model based on adult IFNAR $^{(-/-)}$ mice that support the in vivo growth of BTV-4 and BTV-8 after intravenous inoculation. A tremendous advantage of this mouse model is the availability of a wide variety of reagents that can be used to study many aspects of the immune response to the virus. In addition, we propose this animal model as an adequate system for testing BTV vaccines, an important issue because the cost of testing new vaccines in target species is a major obstacle for laboratories and industries. This mouse model for the study of BTV infections has unique features that open the possibility to study in the same host-virus system susceptibility, virulence, immunobiology of infection, and vaccine efficacy, in ways that are not approachable with the natural host.

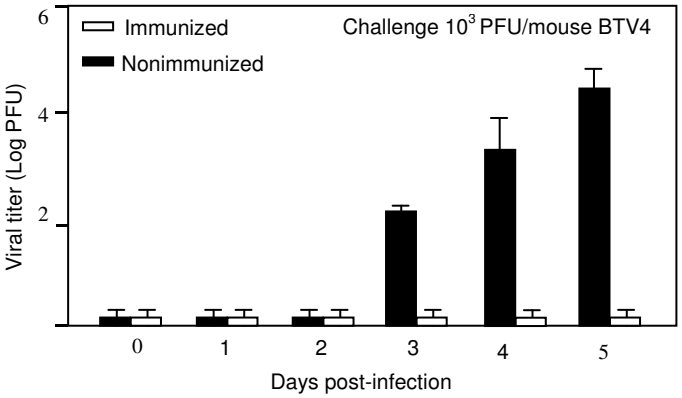

Figure 1. Titers of BTV-4 recovered in blood of immunized and nonimmunized IFNAR$^{(-/-)}$ mice after challenge with BTV-4 (10^3 PFU/mouse). Virus was extracted from blood and determined as described in Materials and Methods. Each point represents the mean values of the viral titer of eight animals, and standard deviations are shown as error bars.

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HETEROLOGOUS PRIME BOOST VACCINATION WITH DNA AND RECOMBINANT MODIFIED VACCINIA VIRUS ANKARA EXPRESSING VP2, VP5, AND VP7 PROTEINS OF BLUETONGUE VIRUS PROTECTS IFNAR$^{(-/-)}$ MICE AGAINST BLUETONGUE INFECTION

Eva CALVO-PINILLA, Teresa RODRÍGUEZ-CALVO, Noemí SEVILLA, Javier ORTEGO

Centro de Investigación en Sanidad Animal, CISA-INIA, Madrid, Spain

Keywords: bluetongue virus, DNA/MVA vaccines

Introduction and objectives

Bluetongue (BT) is a noncontagious, insect-transmitted disease of certain breeds of sheep and some species of wild ruminants that is caused by BTV. Recent recombinant DNA technology has provided novel approaches to develop marker and safe vaccines against bluetongue virus (BTV). Here, we describe the heterologous prime boost vaccination using DNA and recombinant modified vaccinia virus Ankara (rMVA). We used this strategy using DNA/rMVA expressing VP2 and VP5 proteins (DNA/rMVA-VP2,-VP5) or VP2, VP5, and VP7 proteins of BTV-4 (DNA/rMVA-VP2,-VP5,-VP7) in IFNAR$^{(-/-)}$ mice, the animal model for BTV infection established in our laboratory (1), for its ability to stimulate immune responses and protect against BTV infection.

Materials and methods

Prime boost immunization and challenge with BTV-4 in IFNAR$^{(-/-)}$ mice. Groups of 6 IFNAR$^{(-/-)}$ mice were immunized by heterologous prime boost vaccination with DNAs and rMVAs expressing BTV-4 proteins or phosphate-buffered saline (PBS) (controls), administered 2 weeks apart. A suspension of 50 µg of each pcDNA3 construct was administered intramuscularly and 10^7 PFUs of each rMVA construct were inoculated intraperitoneally. Two weeks after immunization all mice were intravenously inoculated with 10^3 PFUs of BTV-4 (lethal dose). Mice were bled before each immunization and virus challenge. Sera were tested for BTV-4 neutralizing antibodies by Virus Neutralization Test (VNT).

Detection of BTV-4 in blood. Whole blood was collected in EDTA from all animals at regular intervals after inoculation. The viruses were released from whole blood by three freeze/thaw cycles. The amount of infectious virus was measured by plaque assay on Vero cells or RT-qPCR specific for BTV segment 5 (2).

Results

To develop new vaccination strategies against BTV infection we have engineered naked DNAs rMVA expressing VP2, VP5 and VP7 proteins from BTV. IFNAR$^{(-/-)}$ mice inoculated with DNA/rMVA-VP2,-VP5 in an heterologous prime boost vaccination strategy generated good levels of neutralizing antibodies against BTV-4. The appearance of viremia was delayed in vaccinated animals and the highest viremia levels were 10 fold lower than in infected nonimmunized mice. This vaccine conferred partial protection against a challenge with BTV-4. When VP7 was included into the BTV proteins used for immunization, immunized IFNAR$^{(-/-)}$ mice were completely protected against the virus. VP2 and VP7 proteins expressed in the DNA/rMVA vaccines induced a T-cell response that contributed to the protection of IFNAR$^{(-/-)}$ mice against challenge with BTV-4. In addition, the DNA/rMVA-VP2,-VP5,-VP7 vaccine is a marker vaccine. Antibodies against VP2, VP5, and VP7, but not NS3 were detected in the sera of DNA/rMVA-VP2,-VP5,-VP7 immunized mice confirming the DIVA properties of this vaccine.

Discussion and conclusions

A variety of vaccines have been developed to prevent BTV infection, including modified live virus (MLV), inactivated, and recombinant vaccines. MLVs are immunogenic and capable of generating strong protective immunity although they are not safe because of the adverse side effects and the natural transmission of MLV strains from vaccinated to nonvaccinated animals. On the other hand, inactivated vaccines are safe but require repeated immunization because of loss of immunogenicity. In addition, MLV and inactivated vaccines are not marker vaccines. Here, we show that the heterologous prime boost vaccination with DNA and rMVA expressing VP2, VP5, and VP7 proteins of BTV-4 (DNA/rMVA-VP2,-VP5,-VP7) stimulates immune responses and completely protects IFNAR$^{(-/-)}$ mice against BTV-4 infection. In addition, this recombinant marker vaccine allows the distinction between vaccinated and naturally infected animals.

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PRE-INFECTION OF PIGS WITH *MYCOPLASMA HYOPNEUMONIAE* ENHANCES THE PATHOGENESIS OF H1N1 BUT NOT H1N2 EUROPEAN SWINE INFLUENZA VIRUS

Gaëlle KUNTZ-SIMON[1], Stéphane GORIN[1], Stéphane QUÉGUINER[1], Céline DEBLANC[1], Anne GAUTIER-BOUCHARDON[2], Séverine FERRÉ[2], Nadia AMENNA[3], Gérard BÉNÉVENT[4], Pierre ECOBICHON[4], Jean-Marie GUIONNET[4], Nathalie THOMAS[4], Roland CARIOLET[4]

[1]AFSSA, LERAPP, Unité Virologie Immunologie Porcines, Ploufragan, France;
[2]AFSSA, LERAPP, Unité Mycoplasmologie Bactériologie, Ploufragan, France;
[3]Laboratoire de Développement et d'Analyses des Côtes d'Armor, Ploufragan, France;
[4]AFSSA, LERAPP, Service de Production de Porcs Assainis et d'Expérimentation, Ploufragan, France

Keywords: *Mycoplasma hyopneumoniae*, pigs, porcine respiratory disease complex, swine influenza virus

Introduction and objectives

Swine influenza virus (SIV) has been recognized to play an important role in respiratory disease of pigs and contributes to the porcine respiratory disease complex (PRDC) that causes important economic losses in affected swine herds. Three subtypes of SIV – H1N1, H3N2 and H1N2 – are currently circulating in swine populations worldwide. However, various lineages can be distinguished within each subtype depending on the world area. Moreover, while many SIV strains have been isolated from pigs showing clinical respiratory symptoms, subclinical infections frequently occur. Little is known about viral components that could determine the strain virulence, but it is widely recognized that multiple factors, e.g. immune status, age, infection pressure, climatic conditions, housing, may interact to affect the clinical outcome of SIV infection. Observations under natural conditions suggested that concurrent bacterial or viral infections may also impact the severity and course of infection with SIVs (1). However, there are only few study of experimental dual infection of pigs with SIV and other pathogens. In this study we intended to compare i) pathogenesis of both SIV subtypes currently circulating in French pigs, e.g. the European avian-like swine H1N1 lineage and the European human-like reassortant swine H1N2 lineage (2), ii) pathogenesis of these H1N1 and H1N2 SIVs in pigs primarily infected with *Mycoplasma hyopneumoniae*, one of the most frequently identified microbes that are directly related to PRDC.

Materials and methods

M. hyopneumoniae (Mhp, strain 116) was isolated from an outbreak of enzootic pneumonia in France and cultivated in Friis broth medium (FBM) at 37°C (3). Field isolates A/Sw/Cotes d'Armor/0231/06 (H1N1) and A/Sw/Cotes d'Armor/0113/06 (H1N2) were propagated in the allantoic cavity of 9-day-old embryonated chicken eggs at 36°C for 3 days.

Forty 6-week-old SPF pigs were randomly allocated to study groups. Twenty animals were inoculated twice intra-tracheally (2×5.10^8 CCU) with Mhp 116, while 20 animals were inoculated twice intra-tracheally with FBM. Three weeks later (21 days post-inoculation – DPI) 5 Mhp infected animals and 5 mock-infected animals were inoculated intra-tracheally (5.10^5 EID_{50}) with H1N1 (MH1N1 and H1N1 groups), whereas 5 Mhp infected animals and 5 mock-infected animals were inoculated intra-tracheally (5.10^5 EID_{50}) with H1N2 (MH1N2 and H1N2 groups). Simultaneously, 10 Mhp infected and 10 mock-infected animals were inoculated intra-tracheally with allantoic fluid (M and C groups). The pigs were monitored daily for rectal temperature and clinical signs and were weighted weekly for four weeks. Nasal swabs were taken at 23, 25 and 28 DPI. Blood samples were collected weekly and sera were separated by centrifugation. Pigs from H1N1 and H1N2 groups were necropsied at 28 DPI to evaluate macroscopic and microscopic lung lesions together with Mhp and SIVs detection. Pigs from MH1N1 and MH1N2 groups were similarly necropsied at 29 DPI, pigs from M group at 30 DPI and pigs from C group at 31 DPI.

Sera were tested for antibody against SIV by hemagglutination inhibition (HI) tests. Nasal swab supernatants as well as lung samples and BALFs recovered at necropsy were tested for SIVs by M gene real-time RT-PCR. SIV infectivity titres in nasal swabs were determined by inoculation of serial supernatant dilutions onto MDCK cell monolayers. Mhp was quantified in lung samples by quantitative real-time PCR.

Results

All Mhp inoculated animals began to cough from 2 weeks post-infection. Mhp DNA was detected in all pig lung samples recovered at necropsy in M, MH1N1 and MH1N2 groups, whereas in lower amount in lungs from the MH1N2 group. Microscopically, characteristic lesions of mycoplasmal pneumonia such as peribronchiolar lymphoid cell infiltration, peribronchiolar lymphoid germinal center formation and edema fluid and inflammatory cells infiltration in alveoli were observed in lung lesion of all Mhp infected pigs, whereas more or less pronounced depending on subsequent SIV infection (see below).

H1N1 mono-inoculated animals became infected and shed virus via the nasal route from 2-7 DPI for 5/5 animals. Pyrexia (> 40°C) was only detected at 1 DPI except for 1/5 animal that showed a biphasic profile with pyrexia till 4 DPI. Other clinical signs mainly included lethargy and inappetence but weight loss and respiratory disorders were limited. Recovery was evident after 2 DPI except for 1/5 animal. Gross lesions and H1N1 RNA were restricted to the apical, cardiac and mediastinal lobes of the lungs. Microscopic analyses revealed bronchiolitis specific lesions. At 7 DPI only 2/5 animals exhibited low positive HI titers in sera. In animals infected with Mhp 21 days before infection with H1N1, the clinical disease was much more severe than in pigs inoculated only with H1N1 or Mhp. The percentage of lung lesions was greater, 5/5 animals exhibited enlarged bronchopneumonia lesions and the H1N1 SIV invaded diaphragmatic lobes of the lungs.

H1N2 mono-inoculated animals, as H1N1 infected ones, shed virus via the nasal route, but at 7 DPI the virus was detected in nasal swab surpernatant from only 1/5 animal. Pyrexia was detected from 1-5 DPI with variable peaks for 3/5 animals. Lethargy, apathy, increased respiratory rates and coughing were more pronounced than in the H1N1 group, resulting in higher weight loss and delayed recovery. Macroscopic scores were higher, H1N2 RNA was detected in the diaphragmatic lobes of the lungs and histological analyses revealed interstitial pneumonia lesions and MALT hyperplasia together with bronchiolitis. At 7 DPI, 5/5 animals exhibited positive HI titers in sera. In animals that were previously infected with Mhp, nor the pathogenesis of SIV H1N2, nor that of Mhp by itself, were enhanced.

Discussion and conclusions

In this study, we showed that the European avian-like swine H1N1 virus induced clinical and pathological effects in SPF pigs that clearly differed from those induced by the European human-like reassortant swine H1N2 virus. The H1N1 virus was slightly pathogenic as compared to the H1N2 strain. These results confirmed that SIV pathogenicity may vary depending on the strain subtype, without any involvement of external factors such as herd status or monitoring conditions. They sustained observations made in the field, e.g. some subclinal versus more acute infection with H1N1 and H1N2 viruses, respectively.

In accordance to previous reports (4, 5), dual infections showed that *M. hyopneumoniae* and SIV H1N1 acted as synergistic pathogens with increased clinical signs and lung damages originating from both Mhp and H1N1 single infections. In this study we also first showed that there were no apparent synergy between Mhp and SIV H1N2. Surprisingly, the H1N2 infection could even result in Mhp clearance, perhaps due to induction of more rapid and efficient immune responses.

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PATHOGENICITY OF SELECTED FOWL ADENOVIRUS ISOLATES

Helena GRGIĆ, Dan-Hui YANG, Sheila WATSON, Éva NAGY

University of Guelph, Department of Pathobiology, Ontario Veterinary College, Guelph, Canada

Keywords: fowl adenovirus, inclusion body hepatitis, pathogenesis

Introduction and objectives

Fowl adenoviruses (FAdV) are opportunistic pathogens, shed in the feces, and spread both horizontally and vertically. Some FAdVs are the causative agent of inclusion body hepatitis (IBH). The pathogenesis of the disease following infection by the natural route is not known. Therefore, we investigated the pathogenicity of two fowl adenovirus isolates, a serotype 4 (FAdV-4) and a serotype 8 (FAdV-8) in specific pathogen free (SPF) chickens inoculated by the oral or intramuscular (im) routes.

Materials and methods

FAdV-4 was isolated from broiler breeders with no clinical signs of the disease, while FAdV-8 was isolated from an outbreak in commercial broiler chickens. Pathogenicity was evaluated by daily observation of clinical signs and by gross and histological lesions of the experimentally infected SPF chickens (1,3). We investigated the pattern of virus dissemination in selected organs at various times after inoculation. Viral copy numbers in liver, bursa of Fabricius and cecal tonsils were determined by quantitative PCR (2). Statistically significant P-values of viral copy numbers between different tissues and birds in different groups were based on Kruskal-Wallis test pair-wise comparison (5). Furthermore, virus titres from cloacal swabs collected over the entire study period were compared between the orally and im inoculated chickens (2). Antibody response to FAdV was measured by an adenovirus-specific ELISA (4).

Results

Clinical signs of the disease were not seen in either the FAdV-4 or FAdV-8 groups during the 28-day observation period after inoculation. The classical pathological signs of IBH characterized by focal hepatic necrosis, intranuclear inclusion bodies associated with both FAdV-4 and FAdV-8 or hydropericardium associated with only the FAdV-4 serotype were not noted in any of the infected chickens. The highest number of viral copies, irrespective of the inoculation route for both viruses was detected in the cecal tonsil followed by liver and then bursa. Each virus and each inoculation group (oral and im) were subjected separately to the Kruskal-Wallis test to evaluate differences in copy numbers among tissues. At the group level the FAdV-4 orally-inoculated birds had significantly higher ($P<0.001$) viral DNA copies than the im group. Differences in virus titres from cloacal swabs between the FAdV-4 oral and im group were significant ($P<0.001$) with the oral group having higher titres. For the FAdV-8 group there were significant differences in copy numbers in orally inoculated birds ($P<0.001$) and the im group ($P<0.0012$) throughout the examination period.

Interestingly, for the FAdV-8 groups viral titres in the cloacal swabs were higher in the im inoculated birds compared to oral inoculation.

The antibody response was measured by ELISA, and the titres were significantly ($P<0.001$) different between either route of inoculation and control chickens. The differences in titres between birds inoculated orally and im were significant ($P<0.001$). Chickens inoculated im had higher titres than chickens inoculated orally for both FAdV-4 and FAdV-8 groups.

Discussion and conclusions

Under our experimental conditions neither virus caused clinical disease in SPF layer chickens. However, virus was found in tissues and cloacal swabs in the birds inoculated with either FAdV-4 and FAdV-8, despite a lack of clinical signs and pathological changes. Viral copy numbers in cecal tonsils were significantly different from each other for all groups. Based on virus titres in cloacal swabs, there appeared to be more shedding by FAdV-4 birds inoculated orally compared to im, while for FAdV-8 birds, the im inoculated ones shed more virus than the orally infected ones. However, for both FAdV-4 and FAdV-8, those inoculated im had higher antibody titres than orally inoculated ones. These data are important in developing a strategy for using FAdV-4 or -8 as a vaccine vector.

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SPECIES-SPECIFIC CONTRIBUTION OF THE FOUR C-TERMINAL AMINO ACIDS OF INFLUENZA A NS1 PROTEIN TO VIRULENCE

Sébastien M. SOUBIES, Christelle VOLMER, Guillaume CROVILLE, Pierrette COSTE, Caroline LACROUX, Josianne LOUPIAS, Brigitte PERALTA, Jean-Luc GUÉRIN, Romain VOLMER

INRA, UMR 1225, Ecole nationale vétérinaire de Toulouse, 31076 Toulouse, France

Keywords: duck, influenza, mouse, NS1

Introduction and objectives

Large-scale sequence analysis of influenza viruses revealed that NS1 protein from avian Influenza viruses possess a conserved C-terminal ESEV amino acid motif, while NS1 from typical human Influenza viruses possess a C-terminal RSKV motif. The avian ESEV motif has been shown to increase virulence in mice when introduced into the NS1 of the A/WSN/33 strain, but its role in the pathogenesis of an avian Influenza virus remains unknown. Herein, we assessed the contribution of the C-terminal domains motif of NS1 to the pathogenicity of an avian Influenza in an avian host and a mammalian host. By using reverse genetics, we generated H7N1 viruses containing a NS1 with a C-terminal avian ESEV domain or a C-terminal human RSKV domain and we compared the phenotype of these viruses in ducks and mice.

Materials and methods

The A/turkey/Italy/977/1999(H7N1) virus used in this study was a gift from Dr. I. Capua and Dr. W. Dundon (Instituto Zooprofilattico Sperimentale delle Venezie, Legnaro , Italy). Reverse genetics was performed using a eight plasmid system based on the ambisense phw2000 plamsid (gift from Pr. R. Webster, St Jude Children's Research Hospital, Memphis, USA). Site-directed mutagenesis was performed using the Stratagene Quickchange kit. Viruses were grown in embryonated chicken eggs. Each virus was entirely sequenced to verify the presence of the introduced mutations and the absence of additional unwanted mutations. Viruses were titrated on MDCK cells. In order to analyze the replication of these viruses in cells of avian and mammalian origin, we infected primary duck fibroblasts and murine embryonic 3T3 fibroblasts. *In vivo* experiments were carried out by infecting two weeks old pekin ducks (*Anas platyrhynchos*) orally with 10^7 pfu. Three weeks old Balb/c mice were infected intranasally with 10^5 pfu. Cytokine expression was analyzed by quantitative RT-PCR, except for duck type I interferons which were titrated using a biological assay based on the quantification of luciferase expression under the control of the chicken Mx promoter (gift from Dr. P. Staeheli , Universität Freiburg, Germany)

Results

Our results show that the avian C-terminal ESEV motif of NS1 increases virulence in mice. We could link this increase in pathogenicity to a higher viral replication and a higher production of type I interferon. Interestingly, the human C-terminal RSKV motif of NS1 was found to increase virulence in ducks. Ducks infected with the H7N1 virus possessing a C-terminal NS1 RSKV domain had higher viral load and type I interferon induced genes compared to those infected with the H7N1 virus with a C-terminal NS1 ESEV domain.

Discussion and conclusions

We identify the C-terminal domain of NS1 as a species dependent virulence domain. Intriguingly, the typical avian domain confers higher virulence in a mammalian host, while the typical human C-terminal domain increases virulence in an avian host. Thus, our results suggest that NS1 experiences host dependent adaptative evolution that leads to the selection of a C-terminal domain conferring lower virulence in its natural host.

AVIAN INFLUENZA VIRUSES USE DIFFERENT RECEPTORS FOR INFECTION OF THE RESPIRATORY EPITHELIUM IN DIFFERENT AVIAN HOST SPECIES

Maren BOHM[1], Christine WINTER[1, 2], Georg HERRLER[1]

[1]Institute of Virology, University of Veterinary Medicine, Hannover, Germany;
[2]Clinic for Poultry, University of Veterinary Medicine, Hannover, Germany

Keywords: avian influenza, hemagglutinin, host tropism, tracheal organ cultures, sialic acids

Introduction and objectives

The entry of influenza viruses into the host cell is initiated by the binding of the viral hemagglutinin (HA) to cell surface receptors containing sialic acid as a crucial determinant. The distribution of α-2,3 and α-2,6-linked sialic acids on different cell types and the viral preference for these receptors is believed to determine both the cell and species specificity of the virus.

The HA of avian influenza viruses preferentially binds to sialic acids linked to galactose via an α-2,3-linkage, whereas human influenza viruses preferentially recognize those sialic acids linked to galactose via an α-2,6-linkage. α-2,3-linked sialic acids are abundant in the chicken respiratory epithelium, whereas only present in the lower respiratory tract of humans. α-2,6-linked sialic acids on the contrary are scarcely expressed in the chicken respiratory epithelium but abundant in the human respiratory epithelium (1).

A switch in the likage preference from α-2,3 to α-2,6-linked sialic acids is supposed to be essential for the adaptation of an avian influenza virus to the human host. Therefore, the HA is a determinant of viral host-cell tropism and plays an important role for the viral ability to cross the species barrier and adapt to a new host.

Materials and methods

We have characterized the infection of primary avian respiratory epithelial cells by avian influenza virus strains of the H5, H7, and H9 subtypes. For this purpose, we used tracheal organ cultures (TOCs) from chicken and turkey. TOCs were prepared by manual cutting of the trachea of a 20 days old chick embryo, 25 days old turkey embryo, respectively, in approximately 1mm thick rings. The rings were cultured in an over-head shaker and were vital for up to two weeks.

TOCs preserve the natural arrangement of the epithelial cells. The ciliary activity of the ciliated cells is visible under the light microscope; therefore, an infection by ciliostatic viruses, like influenza viruses, is easily detected by monitoring the ciliary activity.

Results

To analyze the role of sialic acids in the onset of infection, TOCs were pretreated with neuraminidase to remove sialic acids from the cell surface and protect the cells from virus infection.

Viruses containing an H5 or H7 hemagglutinin responded as expected, i.e the enzymatic pretreatment prevented or retarded the ciliostatic effect of the influenza virus infection.

By contrast, neuraminidase treatment did not have any protective effect on the ciliostasis induced in chicken TOCs by an H9N2 strain. On the other hand, infection of turkey TOCs by the H9N2 virus was neuraminidase-sensitive.

To investigate the differences between the two avian species in more detail, we visualized α-2,3 and α-2,6-linked sialic acids on the cell surface by fluorescent staining of cryosections derived from TOCs using specific lectins. The *Maackia amurensis*-agglutinin specifically recognizes α-2,3-linked sialic acids, whereas the *Sambucus nigra*-agglutinin detects sialic acids attached in an α-2,6 linkage.

This staining revealed that respiratory epithelial cells from both chicken and turkey contain α-2,3-linked sialic acids; however, α-2,6-linked sialic acids were found only on the surface of the turkey respiratory epithelium, but not on the epithelial cells from chicken.

Discussion and conclusions

The protective effect of the neuraminidase treatment on the ciliostasis induced in chicken TOCs by influenza viruses containing H5 or H7 confirms the binding of these viruses to α-2,3-linked sialic acids.

In contrast, the neuraminidase resistance of the H9N2 virus infection in chicken TOCs indicates that in chicken this virus prefers another type of receptor that is neuraminidase resistant. This receptor might be a neuraminidase-resistant sialic acid or another type of sugar or even a protein.

The neuraminidase sensitivity of the H9N2 virus infection in turkey TOCs, on the other hand, shows that in this species the virus preferentially recognizes a neuraminidase-sensitive sialic acid.

The difference in receptor binding of the H9N2 virus in chicken and turkey might be related to the lack of the preferred receptor in one of the species. Since H9N2 influenza viruses have previously been described to show a human virus-like binding preference (2), we favor the possibility that α-2,6-linked sialic acids, that were only detected in turkey respiratory epithelium but not in chicken, were bound by the H9N2 virus strain with a high affinity. The absence of α-2,6-linked sialic acids in chicken TOCs results in the attachment of the H9N2 virus to the host cell via an neuraminidase-resistant receptor. The type of this alternative receptor remains yet to be elucidated.

These findings indicate that avian influenza viruses use different receptors on their host cells depending on both the subtype of the hemagglutinin and the host species.

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ASSEMBLY AND MATURATION OF PESTIVIRUSES

Stefanie SCHMEISER*, Matthias KÖNIG, Heinz-Jürgen THIEL

Institut für Virologie, FB Veterinärmedizin, Justus-Liebig-Universität Giessen, Germany;
**Current contact address: Institute of Virology, University of Veterinary Medicine Hannover Foundation, Germany*

Keywords: electron microscopy, immunogold labelling, pestiviruses, time course studies, virion assembly

Introduction and objectives

The virus family *Flaviviridae* contains the three genera *Hepacivirus*, *Flavivirus* and *Pestivirus*. Members of the genus *Pestivirus* are bovine viral diarrhea viruses (BVDV-1 and BVDV-2), classical swine fever virus (CSFV) and border disease virus (BDV), all of which have a significant economic impact on farming worldwide. Because of their close relationship to Hepatitis C virus (genus *Hepacivirus*), pestiviruses are studied as a model for this important human pathogen.

Pestivirus particles are enveloped with a size of 40-50 nm and contain a single stranded RNA genome of positive polarity. Virions (fig. 1) have been proposed to show icosahedral symmetry. In ultrathin sections of infected cells virus particles exhibit a hexagonal outline of the envelope [1]. The viral capsid is composed of a single core protein (C) and the viral RNA. The symmetry of the viral capsid has not yet been determined. Three glycoproteins designated E1, E2 and E^{rns} (E- envelope; rns – ribonuclease secreted) are associated with the host cell derived lipid membrane forming the viral envelope (fig. 2).

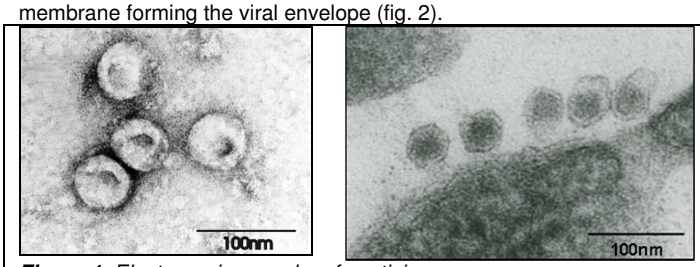

Figure 1. *Electron micrographs of pestiviruses.*
Left: *Negative staining of purified pestivirions.*
Right: *Thin section of infected MDBK cells with extracellular virions.*

Morphogenesis of pestiviruses is poorly understood. Assembly of virions takes place by budding presumably at ER-derived intracellular membranes. It is assumed that virions are transported via the membrane systems of the host cell secretory pathway to the cell surface and are released by exocytosis. In contrast to other virus systems studies on morphogenesis of pestiviruses at the ultrastructural level are impeded by the low rate of virion synthesis. There is currently only one report demonstrating pestivirus particles inside ER structures by transmission electron microscopy (TEM) [2].

The objectives of our study were to localise pestivirus assembly and investigate subsequent maturation at the ultrastructural level in infected culture cells. In addition alterations of intracellular membranes after infection with pestiviruses should be studied. Membrane alterations have been shown in cells infected with Kunjin virus of the genus *Flavivirus* [3] and in cells expressing parts of the Hepatitis C Virus polyprotein [4].

Materials and methods

Different pestivirus isolates (Giraffe-1, BVDV-2 890, CSFV Alfort-Tübingen) and cell lines (MDBK, PK-15, SK-6) were selected. The optimal timeframe for ultrahistological examinations was determined using virus growth kinetics and quantitative real-time RT-PCR. Conventional embedding technique (epoxid resin) was applied to study cellular morphology and virion localisation. The Tokuyasu technique modified after Liou et al. [5, 6] was applied to perform single and double immunogoldlabeling studies on cryo thin sections for studies on pestivirus protein distribution within the infection culture cells.

Results

Virus growth kinetics showed that initial virus replication and release take place between 10-16h after infection. This early time frame was chosen for ultrastructural examination to avoid effects of viral cytopathogenicity and secondary infections. Infected cells embedded in epoxid resins contained low numbers of virions in various cellular compartments including rER, Golgi-Complex and small vesicles. The process of virus release could be observed eventually. Detection of virions inside multivesicular bodies (MVB) could not be verified at this stage; structures similar to virions were also observed in MVBs of non infected control cells. Interestingly no significant alterations of intracellular membranes were observed in numerous experiments.

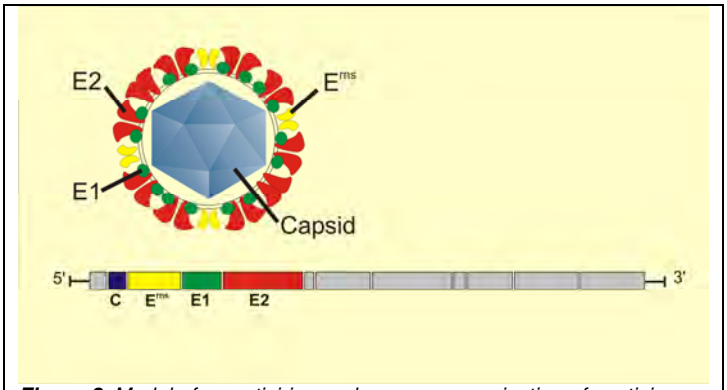

Figure 2. *Model of a pestivirion and genome organisation of pestiviruses*

The immunogoldlabelling on cryosections was used to determine the distribution of pestiviral protein in infected cells. The presence of core protein and E2 at ER membranes was proven by colocalisation with the ER marker protein disulfide isomerase (PDI). The E^{rns} protein was not detected earlier than 24h p.i. (non cytopathogenic BVDV-2 890) when specific labelling was observed inside multilamellar bodies (MLB). In addition labelling for core and E2 protein could be shown inside multivesicular bodies which belong like the MLBs to the endosomal compartment. These results support the morphological findings obtained by conventional embedding techniques.

Discussion and conclusions

Ultrastructural examinations substantiate the current model of pestiviral morphogenesis including the release of virions from infected cells. Despite extensive examination of infected cells no significant membrane alterations could be observed. Virions were found inside clearly identifiable ER structures making it highly unlikely that rearrangements of cellular membranes are required for pestivirus morphogenesis.

By immunogold labelling on ultrathin cryosections of infected cells intracellular distribution of pestivirus proteins was analyzed at the ultrastructural level for the first time proving localisation of structural proteins at the ER membranes. In addition structural proteins and virions were found inside MVBs of the endosomal compartment. To integrate the latter observation into the current concept of pestiviral morphogenesis. further investigations are needed.

Acknowledgements

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EXPERIMENTAL INFECTION OF CALVES WITH BTV-8 USING TWO DIFFERENT INOCULA

Ludovic MARTINELLE[1*], Fabiana DAL POZZO[1*], Pierre SARRADIN[2], Ilse DE LEEUW[3], Kris DE CLERCQ[3], Dominique ZIANT[1], Etienne THIRY[1], Claude SAEGERMAN[1]

[1]Faculty of Veterinary Medicine, Department of Infectious and Parasitic Diseases, Liège, Belgium; [2]INRA UE 1277, Plate-forme d'Infectiologie Experimentale, Nouzilly, France; [3]Veterinary and Agrochemical Research Centre, Virology Department, Brussels, Belgium;
** Both authors contributed equally*

Keywords: bluetongue virus, calves, experimental infection, serotype 8

Introduction and objectives

Cattle are commonly subclinically infected following natural or experimental infection with bluetongue virus (BTV). The introduction of BTV serotype 8 (BTV-8) in Europe (2) has been characterized by the manifestation of clinical signs in infected cattle (3). In order to study the pathogenesis of BTV-8 in this host, an animal model able to reproduce the clinical manifestations of the disease is required. In the OIE *Terrestrial Manual*, the use of wild type virus with no cell-culture passage in the course of challenge infections with BTV is recommended. However, during a preliminary study with BTV-8, we could successfully reproduce clinical signs of the disease in calves infected with a low cell-passage virus (1). Considering the difficulty to generate clinical manifestations of the disease under experimental conditions and the need to have a highly reproducible and reliable model, an extensive study has been implemented in order to compare two different inocula. The aim of this work was to investigate the effects of low cell-passage virus and infectious blood on the evolution of the disease in experimentally infected calves.

Materials and methods

Twelve female Holstein calves between 5 and 6 months old have been used. They were bought in a French BTV-free area before the beginning of the experiment and were tested negative for BTV. In addition, they have been also tested negative for bovine viral diarrhea virus (BVDV) and infectious bovine rhinotracheitis virus (BoHV-1). Calves were confined in an insect-secure zone at the Experimental Infectiology Platform (PFIE) of the INRA centre of Tours (Nouzilly, France). They were separated in three physically isolated groups: group A (five animals infected with low cell-passage virus), group B (five animals infected with infectious blood) and group C (two animals, non-infected). One week before the infection, daily body temperatures were recorded and haematological analyses were realized as reference basal values. BTV-8 strain BEL2006/01 P2 with a titer of $10^{4.5}$ ELD_{50}/ml was used as cell-passage virus. Infectious blood obtained in the course of a preliminary experiment from an infected calf during the viraemic peak (1), had a titer of 10^{3} ELD_{50}. In both groups of infected calves, the administration route of the virus inoculum was strictly intravenous (jugular vein). The animals were monitored daily by a veterinarian during the entire course of the experiment. The body temperature and the development of clinical signs were examined daily and at the same moment of the day (in the morning), and a digital camera was used to record the evolution of the lesions. A standardized clinical form for BTV infection was used during the animal examination (3). A total clinical score was calculated for each animal daily, at the end of the experiment and for each group of clinical signs. The animal management was done starting always from group C, followed by group A and group B. EDTA-blood and sera were collected during the entire experiment. A daily haematological analysis was performed on newly collected EDTA-blood (ABC Vet, SCIL animal care company). After 46 days post-infection, animals were euthanized following an IV injection of pentobarbital. The dynamic of viraemia was investigated using EDTA-blood samples daily collected during the experiment. Viral RNA was detected by a qPCR (5), using BTV segment 5 as the target gene during amplification. Bovine beta-actin was contemporarily amplified as an internal control in order to identify the presence of inhibitors. Quantification cycles (Cq) for each sample were used to compare the daily blood RNA viral load. Furthermore, BTV was isolated on embryonated chicken eggs (ECE), in order to define the interval characterized by the presence of viable infectious virus in the bloodstream. Blood sera were collected and stored at –20°C. A competitive ELISA for the detection of BTV specific antibodies was used (ID Screen® Bluetongue Competition ELISA kit, ID VET, Montpellier, France). The cELISA was performed following manufacturer's instructions.

Results

While the two non-infected calves did not show any clinical signs of infection and remained healthy during the entire experiment, animals in both groups A and B showed clinical conditions compatible with bluetongue disease. Mild pyrexia was observed in two animals of group A and B 2-4 days PI. An increased body temperature compared to the basal values recorded during the acclimatization week was observed at 7-10 days PI involving all the animals of group B and 4 on 5 of group A. The calves of both groups showed oral inflammation of various kinds, including reddening of the region around the lingual frenulum, red patches on the dental pad and the hard palate, ulcers, general erythema and oedema (more noticeable on the gum). Lesions evolved very often on the same pattern with an initial reddening of the frenulum of the tongue, followed by oedema of the gum and reddening of the inside face of the cheeks. In calves of group A, lesions appeared to be less severe, mostly characterized by erythema and swelling of different levels in the mouth. Later lesions, beginning at 14 days PI, occurred mainly under the form of crusts on the dental pad, the nostril and the muzzle, and the lower lip. In the acute phase of the infection, total clinical score of calves in group B was higher than in group A whereas lately (after 14 days PI), total clinical score of animals in group A went slightly higher than group B. In animals of group B, BTV RNA could be amplified only after day 7 PI, whereas in most of group A it could be detected as soon as 3 days PI. In both groups the lower Cq values were measured between day 10 and 12 PI. Afterwards, Cq values increased progressively, but BTV RNA could be consistently detected until the end of the experiment (46 days PI). None of the animals belonging to group C had detectable Cq at any time of the experiment. BTV could be isolated on ECE starting from 7 days PI in group B. Animals of group A mostly seroconverted between day 9 and 10 PI, while calves of group B seroconverted later between day 11 and 24 PI. In calves of group A and B the haematological analyses showed a modified lymphocyte proportion in the whole white cell population compared to the reference values. The lymphocyte proportion rose up 24h PI, reaching a peak 48h PI and then remained stable for 10 more days. White blood cells total count started a slow decrease 24h PI, with a minimum at 8-9 days PI, and then followed an increase overtaking normal values 3 days later. Group C showed significant modifications neither in white blood cells count nor in lymphocyte proportion. The necropsy revealed the presence of a common pattern of lesions in group A and B: petechial haemorrhages were observed in prescapular, mediastinic, mesenteric lymph nodes and thymus. In both groups, some infected calves had pleuritis and exudative pericarditis.

Discussion and conclusions

The two inocula were suitable to reproduce clinical signs similar to the ones related to natural disease. However, differences depending on the used inocula were pointed out. More severe clinical signs were observed and quantified in the acute phase in calves infected with wild type virus. In addition, in the same group of calves, BTV RNA was detected later and it was associated with a later seroconversion. Our results highlighted that both infectious blood and low cell-culture passage BTV-8 could be used successfully in the reproduction of bluetongue disease in experimentally infected calves.

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BOVINE IMMUNODEFICIENCY VIRUS INFECTION FAILS TO PROVIDE PROTECTION AGAINST SUBSEQUENT JEMBRANA DISEASE VIRUS INFECTION

Tegan J. McNab[1], Moira Desport[1], I. W. Masa Tenaya[1, 2], Nining Hartaningsih[2], Graham E. Wilcox[1]

[1]*School of Veterinary and Biomedical Sciences, Murdoch University WA 6150, Australia;*
[2]*Balai Besar Veteriner Denpasar, Bali, Indonesia.*

Keywords: bovine immunodeficiency virus, cattle, Jembrana disease virus, lentivirus, superinfection

Introduction and objectives

Two bovine lentiviruses have been described, *Jembrana disease virus* (JDV) and *Bovine immunodeficiency virus* (BIV). Although genetically and antigenically related, the two viruses differ markedly in pathogenicity. BIV is of minimal pathogenicity: it produces a subclinical infection in *Bos taurus*[1] and *Bos javanicus* (Bali cattle)[2]. In contrast, JDV produces an acute disease in Bali cattle with a case fatality rate of 20%[3,4]. The acute disease occurs 6-14 days after infection and is characterised by a febrile response, leukopenia and lymphadenopathy. During the acute phase of Jembrana disease there is a high plasma viraemia with levels peaking at 10^{12} genome copies/ml plasma and pathological changes that reflect a disease affecting the lymphoid system. JDV causes a milder subclinical infection in *B. Taurus*[4].

The objectives of this study were to re-examine the effects of BIV infection in Bali cattle and to determine whether prior infection of Bali cattle with BIV might ameliorate the effects of subsequent JDV infection. Recent studies in domestic cats have shown that infection with non-pathogenic lion and puma lentiviruses can partially protect against superinfection with virulent feline immunodeficiency virus[5].

Materials and methods

Fifteen Bali cattle were infected with 1.38×10^6 $TCID_{50}$ of the R-29 strain of BIV. Nine of these cattle were superinfected 42 days later with approximately 1000 ID_{50} of $JDV_{TAB/87}$. An additional 4 cattle were infected with $JDV_{TAB/87}$ only. All cattle were monitored until 30 days after infection with JDV for clinical signs, plasma viral RNA load, provirus within peripheral blood mononuclear cells (PBMC) and an antibody response to the transmembrane (Tm) glycoproteins and capsid (Ca) proteins of BIV and JDV.

Results

BIV infection of Bali cattle was demonstrated by the detection in all BIV inoculated cattle of provirus in PBMC over the course of the experiment and the transient detection of viral RNA in plasma in 8 of 13 inoculated cattle. No significant clinical signs were observed in any of the cattle. There was a rapid antibody response to the Tm and a slightly more delayed response to the Ca protein but in both cases the antibody response occurred much earlier than that which occurs in Bali cattle in response to JDV infection.

Subsequent infection of the BIV-infected cattle with JDV, 42 days after infection with BIV, resulted in a transient reactivation of the BIV viraemia in 4 of 9 cattle but it did not affect JDV replication and it did not result in amelioration of the clinical signs of Jembrana disease in the JDV-infected cattle. After JDV infection, there was a rapid antibody response to the Tm and Ca in the superinfected cattle, compared to that in cattle infected with JDV only.

Discussion and conclusions

There was clear evidence that BIV replicated in Bali cattle and that infection was not associated with clinical signs of disease. The evidence for replication included the detection of BIV provirus in PBMC in all cattle at numerous timepoints throughout infection, and the transient detection of viral RNA in plasma in the period 8 to 13 days after infection. The detection of viral RNA in plasma suggested the occurrence of a transient viraemia soon after infection, similar to that observed in other lentivirus infections.

Prior infection with BIV failed to ameliorate the effects of JDV infection 42 days after the initial infection with BIV. This was in spite of a strong, pre-existing antibody response against BIV Tm and a rapid antibody response to the JDV Tm and Ca after superinfection with JDV. The result was surprising considering the close genetic and antigenic relationship between the two viruses.

Acknowledgements

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POTENTIAL ROLE OF TICKS AS VECTORS OF BLUETONGUE VIRUS

Chantal BOUWKNEGT[1], Piet A. VAN RIJN[2], Jacqueline J.M. SCHIPPER[1], Dennis HÖLZEL[2], Jan BOONSTRA[2], Ard M. NIJHOF[1], Eugène M.A. VAN ROOIJ[3], Frans JONGEJAN[1, 4]

[1]Utrecht Centre for Tick-borne Diseases (UCTD), Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584CL, Utrecht, The Netherlands;
[2]Department of Virology, Central Veterinary Institute (CVI) of Wageningen UR, PO Box 65, 8200 AB Lelystad, The Netherlands;
[3]Present address: Central Laboratory Animal Research Facility, Faculty of Veterinary Medicine, University Utrecht, P.O. Box 80190, 3508 TD Utrecht, The Netherlands;
[4]Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, 0110, Onderstepoort, South Africa

Keywords: bluetongue, overwintering, ticks, transmission

Introduction and objectives

Bluetongue is a non-contagious infectious disease transmitted by the bites of species of *Culicoides*. Bluetongue infects (domestic) ruminants and causes clinical signs ranging from apathy and weight loss to swollen heads, tender feet and death. Bluetongue virus (BTV) is a double stranded RNA-virus with a segmented genome belonging to the genus Orbivirus of the family Reoviridae with 24, or maybe more serotypes. In August 2006, BTV serotype 8 (BTV8) emerged and spread rapidly in North-West Europe, and the disease disappeared in December 2006. Re-emerging of BTV8 in North-West Europe in the summer of 2007 and again in 2008 have raised the question where BTV8 persists during the Winter and Spring. In particular, in a moderate climate with a period with lower temperature far longer than the lifespan of the adult *Culicoides* vector, and a *Culicoides*-free period expanding the viraemic period of BTV8 in ruminants persistence of BT is unsolved. Several hypotheses, like survival of infected *Culicoides*, transovarial transmission in *Culicoides* spp., reactivation of BTV in the ruminant skin, and vertical transmission in ruminants have been postulated[1, 3, 5]. Here we have studied the potential role of ticks as vectors for bluetongue.

Materials and methods

Ticks:

In fact, there are many other viruses belonging to the genus Orbivirus, that are transmitted by ticks[4]. Interestingly, the vector capacity of the American soft tick *Ornithodoros* coriaceus for BTV has already been reported in 1985. As a typical three-host tick, *I.* ricinus feeds in total approximately three weeks divided over three different hosts. The feeding behaviour of ticks on viraemic hosts on the one hand and the long-term survival of ticks off-host on the other hand, makes ticks excellent candidates to serve as virus reservoirs. Ticks have a relatively long lifespan. *Ixodes* ricinus, the predominant ixodid tick in North-West Europe, feeds on a broad range of hosts including domestic ruminants and takes two to three years to complete its lifecycle. Other ticks, such as *Dermacentor* species complete one generation per year.

Feeding and testing:

We have fed by capillary feeding or by feeding on artificial membranes four species of ixodid ticks (*Ixodes* ricinus, *Ixodes* hexagonus, *Dermacentor* reticulatus and *Rhipicephalus* bursa) and one soft tick species, *Ornithodoros* savignyi with BTV8-containing blood. Uptake of BTV8 by ticks was tested by an in-house developed reverse transcriptase real time PCR assay[3].

Results

Here we have studied the potential role of tick species ixodid endemic in Northern Europ, and argasid ticks as reservoir and transmitters of BTV8. BTV8 was able to pass through the gut barrier and spread via the haemolymph into the salivary glands, ovaries and testes. BTV8 was detected in various tissues of ixodid ticks for up to 21 days post feeding and in Ornithodoros ticks for up to 26 days. It was found after moulting in adult *I.* hexagonus and BTV8 was also able to pass through the ovaries into the eggs of an *Ornithodoros* savignyi tick.

Discussion and conclusions

This study demonstrates that endemic ticks can become infected with BTV8. The transstadial passage in hard ticks and transovarial passage in soft ticks suggest that ticks have potential vector capacity for BTV. These findings are a first step in unravel the role of ticks in the epidemiology of BT. Further studies are required to investigate BTV-transmission by infected ticks to domestic livestock. This route of transmission by ticks could provide an additional clue in the unresolved mystery of the epidemiology, and in particularly the overwintering, of BTV8 in Europe.

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DETERMINATION OF THE ROLE OF HARD (IXODID) TICKS IN THE TRANSMISSION OF LUMPY SKIN DISEASE VIRUS IN CATTLE

Eeva S. M. TUPPURAINEN[1], Hein W. STOLTSZ[2], Milana TROSKIE[2], David B. WALLACE[2,3], Chris OURA[1], Philip S. MELLOR[1], Koos (Jaw) COETZER[2], Estelle H. VENTER[2]

[1]Institute for Animal Health, Ash Road, Pirbright, Surrey, GU24 0NF, UK;
[2]Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa;
[3]Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort 0110, South Africa

Keywords: lumpy skin disease virus, ticks

Introduction and objectives

Lumpy skin disease virus (LSDV) belongs to the genus *Capripoxvirus* within the family *Poxviridae* and subfamily *Chordopoxvirinae*. The virus infects cattle and occurs in most African countries and in the Middle East. Lumpy skin disease (LSD) is an economically important disease and therefore it is included in the list of Notifiable Diseases by the World Organization of Animal Health (OIE). The mode of transmission of LSDV has not been fully established. In general, poxviruses have been shown to enter the host through the skin or respiratory tract. Direct contact between infected and susceptible animals is considered to be a relatively inefficient route of the transmission of LSDV. The virus is secreted in saliva, ocular and nasal discharge and semen of infected animals and therefore contaminated food or water and artificial insemination may serve as a source of infection. Several researchers have observed that the occurrence of the disease is closely connected to warm and wet weather conditions and the abundance of insects. However, little is known about the importance of the different insect vectors in the transmission of LSDV during the natural outbreaks. So far no studies on the potential role of hard ticks in the transmission of LSDV have been carried out.

The aim of this project was to investigate the potential role of laboratory-bred ixodid ticks: *Rhipicephalus appendiculatus, Amblyomma hebraeum* and *Boophilus decoloratus* in the transmission of LSDV between cattle. To evaluate the mode of transmission (mechanical, transstadial, transovarial) the presence of live virus or viral antigen was studied in mouthparts, salivary glands, and gut cells of nymphs and adult ticks post feeding on the skin of experimentally infected cattle and in eggs laid by a female ticks previously fed on the skin lesions.

Material and methods

Six cattle were infected with a virulent field isolate of LSDV. Three different tick species (*R. appendiculatus, A. hebraeum* and *B. decoloratus*) at different life stages were fed on the infected animals during the viraemic stage and directly on the skin lesions. Post-feeding, semi-engorged adult male ticks were transferred to the skin of three non-infected, recipient animals to complete their blood-meals. Fully engorged female ticks were allowed to lay eggs. The eggs and the female ticks were then tested for LSDV using a polymerase chain reaction (PCR) method and virus isolation on bovine dermis primary cells. Engorged nymphs were allowed to develop to adults and then tested. The potential development of clinical signs of LSD in non-infected cattle was closely monitored for and skin, blood and serum samples were collected.

Results

Viral DNA was detected in mouthparts, salivary glands and gut of *A.hebraeum,* R. *appendiculatus* and *B. decoloratus* ticks and in the eggs laid by *B. decoloratus* females previously fed on the skin lesions of experimentally infected animals. Viral DNA was also detected in skin biopsies collected from the feeding sites of infected ticks on the recipient cattle. Also, in non-infected animals a rise in body temperature was detected approximately 6 days post-attachment of infected ticks.

Discussions and conclusions

Previously LSDV has been isolated in the field from *Stomoxys calcitrans* and *Biomyia fasciata* by Du Toit and Weiss in 1960. The mechanical transmission of LSDV by *Stomoxys* flies and *Aedes aegypti* mosquitoes was demonstrated by Kitching and Mellor (1986) and Chihota *et al.* (2000), respectively. However, so far no studies on the potential role of hard ticks in the transmission of LSDV have been carried out. *R.appendiculatus, A. hebraeum* and *B.decoloratus* are common tick species in southern Africa. Although the findings of this study do not indicate that the virus detected from these ticks was still infective, it suggests that the mechanical, transstadial and transovarial transmission of LSDV may occur and consequently, further studies on the role of these ticks as field vectors of the virus are required.

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SEROPREVALENCE OF EQUINE VIRAL ARTERITIS IN CROATIA – VARIABLE SPECIFICITY OF IMUNNOENZIME ASSAY IN DIFFERENT HORSE POPULATIONS

Ljubo BARBIC, Nenad TURK, Zoran MILAS, Vilim STARESINA, Zrinka STRITOF, Josipa HABUS, Vladimir STEVANOVIC, Josip MADIC

Department of Microbiology and Infectious Diseases with Clinic, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

Keywords: ELISA, equine viral arteritis, seroprevalence, specificity, VN-test

Introduction and objectives

Seroprevalence studies that have been performed in different countries shown that prevalence of equine viral arteritis (EVA) infection varies dependent on geographic location and breed of horses (TIMONEY, 2002). Horse population in Croatia could be divided into two significantly different populations regarding management practice. First population is isolated free-range population of horses bred in Lonjsko polje area for meat production. Second population consist of sport and leisure horses frequently transported aboard to tournaments and breeding around Europe where they come in contact with many horses from different European countries. In our study this population was presented by horses from Hippodrome Zagreb. Those two populations haven't had any contact between them and represent distinct epidemiological populations. Studies of presence of EVA in those populations have never been conducted before. In the summer 2008 first confirmed outbreak of EVA in Croatia occurred on State horse farm of Lippizaner, Djakovo and a source of an outbreak is still under investigation (unpublished data). Object of this study was to investigate if EVA was imported for the first time 2008 or is it a wide distributed but not recognized disease in Croatia. At the same time we investigated the specificity of ELISA assay depending on different populations, sexual maturity and gender of tested animals.

Materials and methods

To clarify former EAV prevalence in different horse populations in Croatia we used 200 archive horse sera randomly selected from horse sera collected during 2005. We tested 100 sera from sport and leisure horse population and another 100 from free-range horse populations. Specific antibodies were determinate by ELISA assay (NUGENT et al., 2000) and VN-test (NEWTON et al., 2004). Specificity of ELISA assay depending on population, age and gender was valued in comparation of ELISA results with VN-test results.

Results

Presence of EVA antibodies was confirmed with ELISA assay in 17% horses in free-range population with higher seroprevalence in young than in sexually mature animals. Regarding gender of sexually mature animals the highest seroprevalence were found in stallions. Testing with VN-test showed that all ELISA positive animals in this population were false positive (Table 1). In sport and leisure horse population, seroprevalence determinated with ELISA assay was 12 % with higher value in sexual mature than in young animals. Regarding gender the highest ELISA seroprevalence in sexual mature animals from sport and leisure horse population was found in stallions (Table 1). ELISA positive animals were retested with VN-test. VN-test results showed that seroprevalence in sport and leisure horse population was 9%. All VN-test confirmed seropositive animals were older than 4 years and regarding gender the highest seroprevalence was found in stallions. In contrast to 100% of unspecific positive results in free range population, ELISA assay had 75% specificity in sport and leisure horse population. Regarding age, 100% unspecific positive ELISA assay results were obtained in young animals and specificity in sexual mature animals was 81.7%. Regarding gender in sport and leisure horse population ELISA specificity was 66.7% in mares, 80% in horses and 100% in stallions (Table 1).

Discussion and conclusions

Results of our study showed that EVA was present in Croatia 2005. Disease was present in sport and leisure population, the same population in which outbreak was confirmed in 2008. In opposite the free range horses population was disease free. These results are in accordance with widespread dissemination of EVA at racetracks (McCOLLUM and SWERCZEK, 1978; TIMONEY, 2002). In sport and leisure horses population EVA VN-test confirmed seroprevalence of 9%. Regarding age, higher seroprevalence was found in sexually mature animals than in young animals. This could be result of presence of persistently infected carrier stallions. This conclusion is also supported with results of EVA seroprevalence regarding gender with significantly higher seroprevalence rate in stallions than in mares and castrates. ELISA specificity results showed that all 17% ELISA positive animals in free range population were false positive. ELISA specificity in sport and leisure horse population was in total 75% which is significantly lower than described before by NUGENT et al. (2000). ELISA specificity in this population varied regarding age and sex of tested animals. In sexually mature animals, older than 4 years, ELISA specificity was 81.7%, and all younger animals were false positive. Regarding sex ELISA specificity varies from 66.7% in mares to 100% in stallions. The highest specificity in stallions could also be result of persistently infected carrier status of stallions.

Table 1. *Seroprevalence of EVA in horse populations in Croatia determinate with ELISA and VN-test and ELISA specificity.*

		Age		Gender			Total
		0-4 years	≥ 4 years	Mares	Castrates	Stallions	
Sport and leisure horse population	ELISA positive	6,3%	13,1%	11,1%	11,1%	25%	**12%**
	VN – test positive	0%	10,7%	7,4%	8,9%	25%	**9%**
	ELISA specificity	0%	81,7%	66,7%	80,2%	100%	**75%**
Free range horse population	ELISA positive	23.1%	10.4%	11,4%	0%	63,6%	**17%**
	VN – test positive	0%	0%	0%	0%	0%	**0%**
	ELISA specificity	0%	0%	0%	0%	0%	**0%**

In conclusion, EVA is present in sport and leisure horses in Croatia for at least 4 years, but until 2008 was unrecognized. Results of this study also showed that ELISA specificity varies widely regarding population, age and gender of tested animals. This confirms that ELISA can be used only as a screening method and that VN-test is still the "gold standard" for serology diagnostic of EVA. This study also suggests that different EVA screening and control methods may be requested for different horse populations. Unspecific positive ELISA assay results regarding population/breed weren't described before and indicate some kind of unspecific cross-reaction in Croatian free range horse sera which will be further investigated.

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IDENTIFICATION OF CANINE CORONAVIRUS TYPE I IN A NATURALLY INFECTED CAT

Sophie LE PODER[1], Lidia DUARTE[1], Annie FOURNIER[1], Cristina REBEGA[2], Carine PINHAS[1], Marc ELOIT[1]

[1]UMR 1161-Virologie, INRA, ENVA, AFSSA, Ecole Nationale Vétérinaire d'Alfort, France;
[2]Ecole Nationale Vétérinaire de Iasi, Roumanie

Keywords: cat, coronavirus, dog, interspecies transmission

Introduction and objectives

Coronaviruses have evolved through accumulation of point mutations, insertions, deletions or recombination events within their genome that have led to change in virulence, tissue and/or species tropism. Among cat and dog populations, feline (FCV) and canine (CCV) coronaviruses are widespread. Both FCV and CCV cause mild to severe diarrhoea, especially in young animals, and in approximately 5 per cent of infected cats, FCV causes also a fatal immune-mediated disease called Feline Infectious Peritonitis. The close genetic relationship between feline and canine coronaviruses leads to a potential for cross-species infection. Two genetic clusters are now described among FCV and CCV strains. The FCV type II strains arose from a homologous RNA recombination between CCV and FCV type I (2). Conversely, CCV type I displays some similarity with FCV I in the M, N, ORF-1a, ORF-1b genes, and slightly higher in the S gene, where there is more than 77% identity to FCVI (4). Recently, an additional ORF named ORF3 that was not detected in CCoV type II and other group I coronaviruses was described in CCoV type I (3). The present study was designed to find out whether interspecies transmissions and recombinations between FCV and CCV occurred in the field. Thus, we sequenced a fragment of the nucleocapsid N gene from coronavirus infected cats living or not with a dog. This allowed us to detect a CCVI strain in one cat living in the same household with a dog. We confirmed the identification of CCVI strain by amplification of the typical ORF3 gene.

Material and methods

Clinical specimens: Rectal swab and plasma samples from 32 healthy cats and 6 ascitic fluid samples from cats having clinical signs of a wet form of FIP were tested for the presence of coronavirus. We also collected rectal swabs from the dogs living with the cats studied.

RNA isolation: Viral RNA was extracted from clinical samples using the RNA plus kit (Qbiogene)

Amplification by RT-PCR: Coronavirus detection was performed by using the primers previously described (1). For the amplification of the N gene, PCR primers were chosen for conserved sites on the basis of mismatch to other FCV and CCV strains. ORF3 gene was amplified according to the protocol described by Lorusso et al (3).

DNA sequencing: The PCR products were purified from agarose gels using a silica membrane DNA purification kit (Macherey-Nagel). DNA was cloned using the TA cloning system and nucleotide sequencing was performed by automated sequencing at MWG Company.

Sequence analysis: Deduced viral protein sequences were analyzed and aligned using ClustalW (DNAstar programs, Lasergene). Phylogenetic trees were constructed by the neighbour-joining method in Megalign program.

Results

Detection of coronaviruses in clinical specimens: Rectal swabs were collected from 32 healthy cats referred to our laboratory for a survey of FCV prevalence. As the main purpose of this study was to investigate the possibility of coronavirus cross-species transmission between cats and dogs, we collected also rectal swabs from the dogs living with these cats. By using the RT-PCR assay previously described (1), coronavirus RNA was detected only in feces from 4 cats (Cat-22, Cat-33, Cat-57, Cat-60, and Cat-61). All were living with a dog except FCV-33. We tested also 6 ascitic fluid samples from cats suspected of developing FIP and found FCV RNA in all cases (Cat-1, Cat-18, Cat-21, Cat-24, Cat-41 and Cat-74).

Phylogenetic analysis: To examine the possibility of coronavirus cross-contamination of cats with CCV strains, we amplified the N gene and sequenced the PCR products after cloning. Some N genes were not amplified, probably due to viral loads close to the sensitivity threshold of the RT-PCR assay. The tree (fig 1) shows that the N gene sequences of most coronavirus strains cluster into two main clades that correspond to typical FCV and CCV genotypes. Interestingly, the FCV-22 field isolate segregates with CCV strains and displayed high sequence identity to CCVI (fig. 1). We also demonstrated the presence of the ORF3 gene in this isolate, which confirmed that FCV-22 belongs to the CCVI genotype. A CCVI strain was also identified in the dog (dog-1) living with this cat.

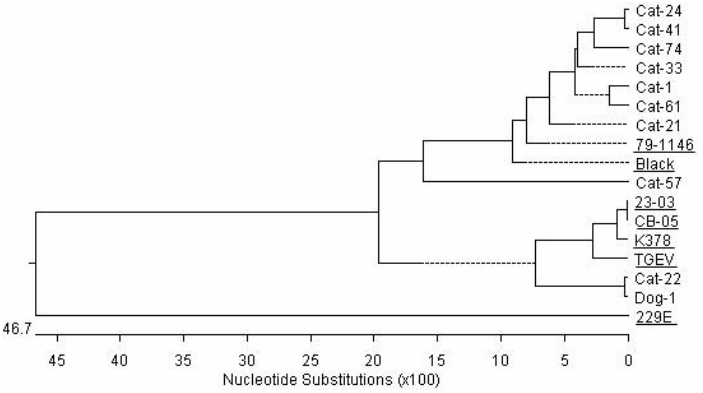

Figure 1. Phylogenetic tree of N gene. Reference strains are underlined.

Discussion and conclusions

The main purpose of this study was to investigate the possibility of interspecific circulation of coronaviruses between cats and dogs. We recruited clinical specimen from cats with FIP symptoms or not, and screened coronavirus positive samples by using a RT-PCR assay specific for the 3'-UTR of the viral genome which is highly conserved in FCV, TGEV and CCV (1). Genotype discrimination of coronavirus positive samples was performed by sequence analysis of N gene. Most of our isolates clustered within the FCV genotype but interestingly the FCV-22 isolate clearly segregated with CCV genotypes. The presence of the ORF-3 gene confirmed that this cat was infected by a CCVI strain. As the same isolate was identified in the sample recovered from the dog living with this cat, we demonstrated in this study that CCVI strains are able to cross the species barrier between dogs and cats. Previous studies have shown that the S protein plays a key role in interspecific transmission. This protein mediates the binding of the virus to the target cell receptor and the subsequent fusion of viral and cellular membranes during entry. The presence of a FCV spike protein in CCoV I may endow the virus with the capacity to enter feline cells. Given the dramatic consequences of SARS, greater understanding of the processes of coronavirus interspecies transmission is crucial. In this context, coronaviruses of domestic carnivores may represent a suitable model to address these questions.

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AN INDIVIDUAL-BASED MODEL FOR PORCINE CIRCOVIRUS TYPE 2 (PCV-2) WITHIN-HERD DYNAMICS OF INFECTION

Mathieu ANDRAUD, Béatrice GRASLAND, François MADEC, André JESTIN, Nicolas ROSE

Agence Française de Sécurité Sanitaire des Aliments (Afssa), 22440 Ploufragan, FRANCE

Keywords: infection dynamics, modelling, PCV-2

Introduction and objectives

Porcine circovirus type 2 (PCV-2) is known as the causative agent of post-weaning multisystemic wasting syndrome.

A stochastic individual-based model representing the population dynamics in a typical French farrow-to-finish pig farm has been developed to study the within-herd course of PCV-2 infection. This model ca be used to study the impact of management practices and vaccination policies on the dynamics of infection within a typical farrow-to-finish farm.

Materials and methods

1. Population dynamics and epidemic model

A stochastic individual-based model was developed to describe the population dynamics within a farrow-to-finish pig farm. This population model was built using a discrete-time simulation approach and has already been described in detail (Andraud et al., 2008b).The epidemiological model is based on a classical SEIR model (Figure 1) with 5 supplementary states representing the effect of passive immunity intake on the infectious process (S_m and P states) and the possibility for the piglets to be infected at birth (states E_n, I_n and I_m). The most important parameters in this epidemiological model are the transmission rates which are dependent on the locations (rooms and pens) and the passive immunity status of each individual. These parameters were derived from three experimental studies which led to the estimation of 6 transmission rates (Rose et al., 2007; Andraud et al., 2008a; Andraud et al., 2009).

2. Strategies and statistical analysis

The model was used to study the impact of different management strategies on the course of infection i.e. cross-fostering, mixing in nursery and size of the pens in nursery which had been identified as major risk factors of clinical PMWS occurrence (Rose et al., 2003). We also investigated the impact of control strategies by using the recently available sow- or piglet-targeted specific vaccines.

The age at infection was studied by recording from simulations the ages at which piglets got the infection. The age-to-infection events were subjected to survival analysis (Proc Lifetest, SAS 9.1). Strategies were tested by comparing survival distributions between strata and modelling the time-to-event using a Cox proportional hazard model with a robust estimate of the variance-covariance matrix to take into account non-independence of pigs within batches (Proc PHREG, SAS 9.1).

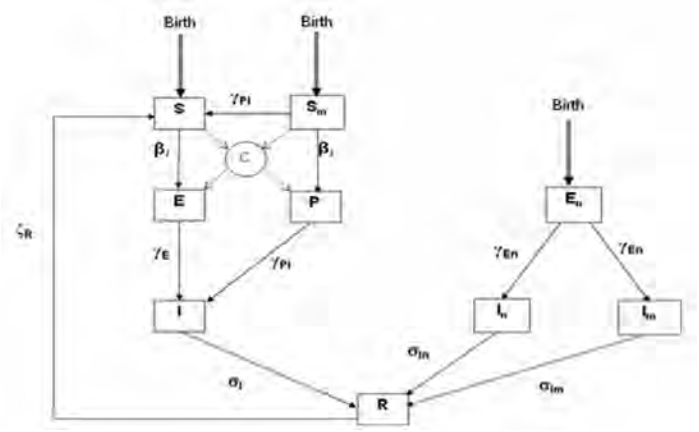

Figure 1: Diagram representing PCV-2 infectious statuses and transitions in sows and growing pigs.

Results

Combination of the 3 management strategies led to 12 different scenarios. The most permissive strategy (random cross-fostering and mixing within large pens in the nursery) was taken as the reference for each comparison. PCV-2 was shown to spread more slowly under all the other tested strategies, the hazard ratios ranging from 1, for the reference strategy, to 0.52 [0.46; 0.59] for the best one (no cross-fostering and grouping piglets by litters in small pens in nursery rooms). However, the risk of early infections was significantly decreased (HR=0.89 [0.80; 0.92]) by modifying just one parameter, such as the size of the pens in the nursery rooms. For the extreme management strategies, the mean seroprevalences were recorded according to dams' parities (parities 1 and 2, 3 and 4 and more than 5) and pig age (8, 13 and 20 weeks old) and were compared with actual seroprevalence data from a field study on risk factors for PMWS. Mean seroprevalences obtained from simulations with the restrictive strategy were close to the observed mean seroprevalence in control and ex-PMWS farms whereas the reference strategy (permissive) led to higher mean seroprevalences in growing pigs indicating a high proportion of early infections, similar to those recorded in herds exhibiting clinical cases of PMWS (Figure 2).

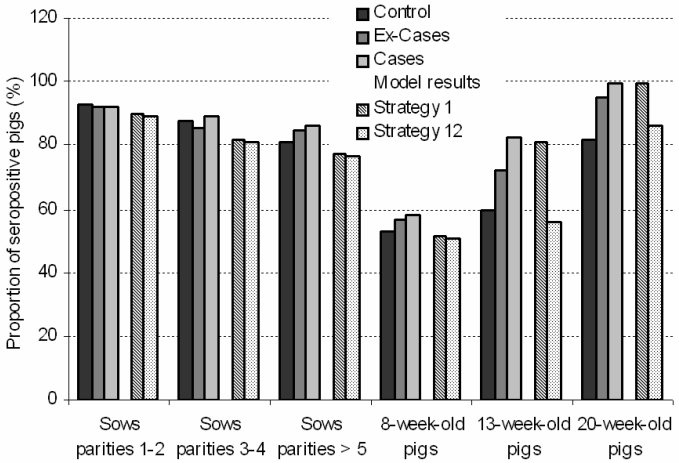

Figure 2: Comparison of simulated seroprevalences according to management strategy with actual seroprevalence data from a field survey (Rose et al., 2003)

Vaccination was shown to significantly reduce the risk of early infections in the reference strategy setting. Sow-targeted vaccine delayed the infectious process until the loss of maternal antibodies, leading to a hazard ratio of 0.49 [0.40; 0.60]. Piglet-targeted vaccine reduced the force of infection and decreased the final number of infected animals (hazard ratio: 0.44 [0.37; 0.54]). However, the restrictive management strategy significantly improved the effects of both vaccination schemes providing hazard ratios of 0.35 and 0.24 for sow- and piglet-targeted vaccines respectively.

Discussion and conclusions

Parameters of this epidemic model were mainly derived from experimental data and simulation results were compared to external field data for validation. The infectious process was shown to be delayed and the number of early infections significantly reduced when the cross-fostering rate was reduced and the pigs were grouped by litters in the nursery rooms. Simulation results showed also that vaccine-based passive immunity was able to delay the infectious process at the end of the nursery phase, thereby decreasing the probability of infection during the lactating phase whereas piglet-targeted vaccines provided active immunity against PCV-2 and greatly reduced the number of infections. The effect of vaccines was greatly influenced by rearing conditions which has to be considered when implementing a prophylaxis program within a PMWS farm.

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INFLUENCE OF SUCKLING PIG CONVALESCENCE FROM INFECTIOUS DIARRHEA ON THEIR SUSCEPTIBILITY TO RESPIRATORY PATHOGENS DURING GROWING PERIOD

Sergey A. KUKUSHKIN, Taufik Z. BAYBIKOV

FGI "Federal Centre for Animal Health" (FGI "ARRIAH"), Department for Monitoring of Porcine Diseases, Vladimir, Russia

Keywords: diarrhea, pig, respiratory pathogens

Introduction and objectives

According to data of Eich K.-O. and Schmidt U. [1] the general mortality level on farms among piglets of all age groups averages 14%; 70% of piglets die during the first week of life. Among infectious diseases of suckling piglets the diarrhea syndrome is responsible for the highest morbidity and mortality level caused by viruses (coronaviruses, rotaviruses), bacteria (*Escherichia coli*, clostridia, etc.), protozoa (coccidia, balantidia, etc.) and their associations [1, 2]. Our researches are mainly aimed at studying the influence of convalescence of suckling piglets from infectious diarrhea on their susceptibility to respiratory pathogens and major economic factors during the growing period.

Materials and methods

The large-scale pig farm with the full production cycle (total population of 65 thousand pigs), where an outbreak of diarrhea syndrome among suckling piglets was registered, was under our observation for several months. For epidemiological examination results of laboratory testings of pathological samples from sick suckling piglets and piglets from the growing group and major economic factors (morbidity, mortality, average daily weight gain, etc.) were analyzed.

Results

In spring 2008 the mass morbidity and mortality among 11-14-day-old piglets with a diarrhea syndrome were registered on the large-scale pig farm located in the central part of Russia; later piglets became affected on day 6-8 after birth. The survival level of suckling piglets on the farm decreased up to 70-73% as compared with 93% before the outbreak. In some sectors the survival level was 50% of the total number of born piglets. As a result of testings held in several diagnostic laboratories porcine epidemic diarrhea virus, porcine rotavirus, *Clostridium perfringens* types C and A, enteropathogenic *Escherichia coli* with adhesins K 88(F4) and K 99(F5) were detected in pathological samples from sick and dead piglets. Raw materials used for feed production demonstrated the high content of toxin T-2.

Production factors of the growing group before and after the diarrhea syndrome outbreak were analyzed (Table).

As seen from the Table, after the convalescence of suckling piglets from diarrhea (March-April) the average weight of one animal at introduction for growing (42-day-old), average daily weight gain and survival rate during the growing period significantly decreased from 10,50±1,40 kg to 7,61±0,55 kg, from 420,72±20,71 g to 325,75±6,56 g and from 84,76±2,94% to 73,44±1,29%, correspondingly. As a result of clinical examination of growing group piglets, recovered from diarrhea, significant number of animals with a respiratory syndrome (suppression, cough, serous nasal discharge, weight loss) was observed. First clinical signs appeared by 60-day-age and in some sectors piglets became sick already by 45-47 days of life. Genomes of European genotype PRRS virus and *Pasteurella multocida* type D were detected by PCR in pathological material samples from emergently slaughtered and died piglets from the growing group. Microbiological testings revealed *Actinobacillus pleuropneumoniae* culture pathogenic for white mice.

Discussion and conclusions

The studies held show the significant influence of convalescence of suckling piglets from infectious diarrhea on their susceptibility to respiratory pathogens and main economic factors (average daily weight gains and survival) during the growing period.

Acknowledgements

The authors gratefully acknowledge field veterinarians from farm. Without their cooperation these studies would not have been possible.

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Table 1. Production factors of the growing group before and after diarrhea outbreak in suckling piglets

Period	*Month*	*Introduced for growing, number of animals*	*Average weight of one animal at introduction for growing, kg*	*Average daily weight gain during growing period, g*	*Survival during growing period, %*
Before diarrhea outbreak in suckling piglets	*January*	*1855*	*11,9-13,0*	*405-480*	*86-92*
		(4 sections)	*12,22±0,52*	*427,50±35,58*	*88,05±3,10*
	February	*2246*	*8,4-10,2*	*421-423*	*83-84*
		(4 sections)	*9,30±0,73*	*422,90±1,98*	*83,25±0,50*
	March (I)	*2406*	*9,6-10,3*	*395-420*	*82,5-83,5*
		(4 sections)	*9,98±0,38*	*411,75±11,35*	*83,00±0,41*
	Total:	***6507***	***10,50±1,40***	***420,72±20,71***	***84,76±2,94***
After diarrhea outbreak in suckling piglets	*March (II)*	*1824*	*8,0-8,3*	*325-340*	*73-75*
		(3 sections)	*8,13±0,15*	*331,33±7,77*	*74,00±1,00*
	April	*2582*	*6,7-7,7*	*319-325*	*71,5-75,0*
		(5 sections)	*7,3±0,44*	*322,40±2,79*	*73,1±1,43*
	Total:	***4406***	***7,61±0,55***	***325,75±6,56***	***73,44±1,29***

Mean value = M±S

DETECTION OF SIX HONEYBEE VIRUSES IN COLONIES AFFECTED WITH COLONY COLLAPSE DISORDER

Ivan TOPLAK[1], Danijela RIHTARIČ[1], Peter HOSTNIK[1], Jože GROM[1], Magdalena VIDMAR[2]

[1]*University of Ljubljana, Veterinary Faculty, Institute for Microbiology and Parasitology, Virology Unit, Gerbičeva 60, SI-1115 Ljubljana, Slovenia*
[2]*University of Ljubljana, Veterinary Faculty, Institute for Breeding and Health Care of Wild Animals Fishes and Bees, Gerbičeva 60, SI-1115 Ljubljana, Slovenia*

Keywords: honeybee viruses, colony collapse disorder, RT-PCR

Introduction and objectives

The honeybee, *Apis mellifera L.,* plays a vital role in agriculture by assisting in the pollination of wide variety of crops. Among pathogens attacking honeybees, viruses are probably the least understood because of lack of information about the dynamics underlying viral disease outbreaks. In years 2007-2009, the increased deaths in bee colonies have been recognised also in Slovenia and could be linked to new phenomenon named colony collapse disorders (CCD). Recently the presence of Israeli acute paralysis virus (IAPV) has been strongly correlated with CCD. In the present study we investigated 18 honeybee colonies by RT-PCR method, clinically affected with CCD, to identify the prevalence of six honeybee viruses; acute bee paralysis virus (ABPV), deformed wing virus (DWV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV), IAPV and sacbrood bee virus (SBV).

Materials and methods

Samples of death adult bees were collected between 2007 and 2009 from 18 apiaries and frozen at -70℃ until use. The frozen samples were homogenised in RPMI (Gibco, USA) with homogenizer (IUL masticator). After homogenisation, the suspensions of samples were centrifuged for 15 min at 3.000 x g. An aliquot of supernatant was used for extraction of total RNA using QIAamp viral RNA mini kit (Qiagen, Germany) according to manufacturer's instructions. All RNA samples were tested for the presence of six viruses (table 2). Part of viral genome of ABPV, DWV, CBPC, BQCV, IAPV and SBV was amplified by RT-PCR using specific primer pair (table 1) and One-Step RT-PCR kit (Qiagen, Germany) reagents according to the manufacturer's instructions. The reaction was performed in a total volume of 25 µl as follow: 2 µl of RNA template, 15 µl of nuclease free water, 5 µl of 5 x PCR buffer, 1 µl of 10 mM dNTP mix 10 pmol of specific primer pair and 1 µl of one step RT-PCR enzyme mix. PCR products were visualized in 2 % agarose gel with ethidium bromide. The size of each PCR product was compared to the 100-bp DNA ladder and results for each virus were interpreted as positive or negative according to the expected DNA fragment (table 1).

Table 1. Primers used for PCR diagnosis

Virus	Forward primer	Reverse primer	Length (bp*)	Reference
ABPV	ABPV 1	ABPV 2	398	(1)
DWV	DWV-F	DWV-R	504	(4)
CBPV	CBPV-F	CBPV-R	455	(6)
BQCV	BQCV-F	BQCV-R	700	(2)
IAPV	IAPV-F	IAPV-R	475	(5)
SBV	SBV-F	SBV-R	824	(3)

*Length of DNA fragment amplified

Results

18 apiaries from Slovenia (*Apis mellifera carnica*), affected with CCD, were tested for the presence of known bee viruses. Five honey bee viruses were detected using specific RT-PCR. Tested samples were found to be positive for ABPV, DWV, CBPV, BQCV and SBV. BQCV was present in 100 % of tested samples, the second most prevalent viruses were DWV (83,3 %) and CBPV (83,3 %), following ABPV with 66,6 % of positive samples. SBV was found only in one sample (table 2). Nucleic acid of IAPV was not detected. In the most of affected colonies at least three viruses were found but in some individual samples we detected one to five different bee viruses.

Discussion and conclusions

The increased numbers of *A. mellifera* L. bee colony collapses in several European countries during the last decade has resulted in great interest in honey bee toxicology and pathology. In last few years, the diagnostic method, for honeybee viruses changed from serological to PCR-based methods. Two out of six examined viruses, CBPV and SBV, produce clinical signs that are clearly identified by beekeepers, whereas the majority of other four viruses are believed to cause persistent infection in honey bees. Of the six viruses identified by PCR in this study, BQCV was detected in all of 18 apiaries, but other two viruses (CBPV and DWV) with very high prevalence (83,3 %) were found.

Table 2. Results of detection of six honeybee viruses (ABPV, DWV, CBPC, BQCV, IAPV and SBV) by specific RT-PCR in Slovenia

Sample name/year	*ABPV*	*DWV*	*CBPV*	*BQCV*	*IAPV*	*SBV*
129/2008	-	-	-	+	-	-
3/2009	-	+	+	+	-	-
2/2009	+	+	-	+	-	-
172/2008	-	+	+	+	-	-
164/2008	+	+	+	+	-	-
163-2/2008	+	+	+	+	-	-
163-1/2008	+	+	+	+	-	-
165/2008	-	+	-	+	-	-
166/2008	+	+	+	+	-	-
287/2007-1	+	+	+	+	-	-
285/2007	+	+	+	+	-	-
1/2008	-	-	+	+	-	-
287/2007-3	+	+	+	+	-	-
287/2007-2	+	+	+	+	-	-
289/2007	+	+	+	+	-	-
286/2007	+	-	+	+	-	-
289/2007-A	-	+	+	+	-	+
1503/2009	+	+	+	+	-	-
positive / total	*12 / 18*	*15/18*	*15/18*	*18/18*	*0/18*	*1/18*
% of positive samples by RT-PCR	66,6 %	83,3 %	83,3 %	100 %	0 %	5,5 %

Legend:
+ specific length of DNA fragment was amplified (positive result)
- specific length of DNA fragment was not amplified (negative result)

In our study, recently reported IAPV, as pathogen responsible for CCD, was not detected in colonies affected with high mortality. Although the role of IAPV in the pathogenesis of CCD remains unknown the presence of other four bee viruses could have high predictive value for apiaries with high mortality. The apiaries, which are infested with *Varroa destructor* and co-infected with ABPV, BQCV, CBPV and DWV could potentially be with high risk for bee colony collapse.

This is the first report of CBPV and BQCV detection in *Apis mellifera carnica* in Slovenia.

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HONEYBEE VIRUSES IN SPAIN: DIAGNOSIS AND DISTRIBUTION. ARE THEY A PROBLEM?

Deborah KUKIELKA, José Manuel SÁNCHEZ-VIZCAÍNO

Animal Health Department and VisaVet. School of Veterinary Medicine. Complutense University of Madrid (U.C.M)

Keywords: honeybees, real time RT-PCR

Introduction and objectives

Spain is considered the third largest honeybee (*Apis mellifera*) producer country in the world. Problems caused by the different honeybee viruses are considered as one of the main causes of the CCD among others like parasites, bacteria and pesticides.

Prerequisites for controlling honeybee viral infections in Spain are the development of highly specific-highly sensitive diagnostic assays and knowledge on the extent of the distribution of the diseases throughout the country. Knowledge on how much spread the main honeybee viruses (Deformed wing virus (DWV), Black queen cell virus (BQCV), Acute paralysis bee virus (ABPV), Sacbrood virus (SBV), Chronic bee paralysis virus (CBPV), Kashmir bee virus (KBV) and Israeli acute paralysis virus (IAPV)) are in Spain is important to obtain preliminary evidence of the impact that these diseases have on the honeybee industry of the country.

The main aim of this work was to put into practice different real time RT-PCR-based procedures developed or adapted by us to identify these virus infections in Spanish bee colonies. These analysis establish the basis to design and implement effective honeybee viral disease control and surveillance programs in Spain.

Materials and methods

Three conventional RT-PCR to detect and quantify ABPV, CBPV, KBV and IAPV were adapted to real time RT-PCR. Three real time RT-PCR based on SYBR Green were developed to detect and quantify DWV, BQCV and SBV. Samples from 484 bee colonies located in 14 of the 15 regions in which continental Spain is administratively divided were collected and analyzed since 2004 to 2008.

Results

Honeybee viruses were present in all the samples we analyzed. No free virus beehives were founded.

DWV was the most prevalent virus in the assessed samples followed by BQCV and CBPV. Co-infection with two or three viruses was highly detected, mostly between BQCV and DWV.

IAPV was present in Spain in only one sample; this finding contradicts the results from other countries such as USA where it was pointed out as a highly CCD correlated virus.

Discussion and conclusions

The proportion of samples positive to the viral agents assessed here is consistent with estimates obtained from surveys conducted in other countries for DWV, BQCV and CBPV but is different for SBV, ABPV and IAPV.

Current studies are being carried out to correlate the viral load the honeybees present with the appearance of clinical symptoms.

Combination of these studies with others focused on parasites, pesticides and bacteria, is needed to understand the problem of CCD.

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MICROARRAY POTENTIAL IN DIAGNOSIS OF INFECTIOUS DISEASES

Akbar DASTJERDI[1], **Rajesh GURRALA**[1, 2], **Nick JOHNSON**[1], **Sylvia GRIERSON**[1], **Francesca MARTELLI**[1], **Nadia INGLESE**[1], **Falko STEINBACH**[1], **Malcolm BANKS**[1]

[1]*Virology department, Veterinary Laboratories Agency, New Haw, Addlestone, Surrey, KT15 3NB, United Kingdom;*
[2]*Division of Microbial Sciences, Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey, GU2 7XH, United Kingdom*

Keywords: diagnosis, microarray, viruses

Introduction and objectives
Emergence of new viral pathogens and re-emergence of known viruses are a challenge for most diagnostic establishments. Using degenerate diagnostic tools have the potential to overcome the difficulty in identifying novel agents from disease syndromes in such establishments. In this regard, degenerate PCR in conjunction with microarray can serve as a powerful versatile diagnostic tool for virus identification/discovery. Microarray is a platform which consists of an array of thousand of known oligonucleotide probes designed to preferably conserved regions of viruses. The oligonucleotides are chemically bound to a solid matrix. The objective of this work is to show the potential of microarray as a diagnostic tool for investigation of infectious viral diseases.

Materials and methods
The chip is a spotted array consisting of 8729 oligos spanning 29 viruses families. Clinical samples were prepared for the microarray analysis as described (1,2) The labelled dsDNA were applied to the Defra Biochip Version 4.1 microarray chip and incubated overnight. The chip was then washed, scanned and the GenePix Pro software used to extract feature values. The analysis of the values was performed by DetectiV software (3). Microarray findings were confirmed with sequence specific PCRs and DNA sequencing.

Results
We have developed a 9.0K oligonucleotide microarray chip (Fig. 1) as part of a defra-funded project (Defra Biochip). The biochip has proved very succeesful in investigation of several cases of terrestrial and marine animal diseases. A porcine sapelovirus (formerly PEV8) was detected in association with polioencephalitis in pigs in the UK. A rabies virus was detected in the brain of a patient from travel acquired rabies in Belfast, Ireland (Fig. 2). A poxvirus was detected in a striped dolphin with skin lesions and a parvovirus was detected in geese experiencing 75% mortality. An international investigation into encephalitic disease in horses in Israel resulted in the detection on the Biochip of an orbivirus (Table 1); this is the first report of this virus outside southern Africa. A teschovirus (PTV5) was detected from the faeces of a healthy pig in Humberside, UK. A number of other cases are in the pipeline from bats, foxes, red and grey squirrels for investigation, Moreover, The platform was successfully applied to the detection and simultaneous genotyping of lyssaviruses (1) and classical swine fever virus isolates.

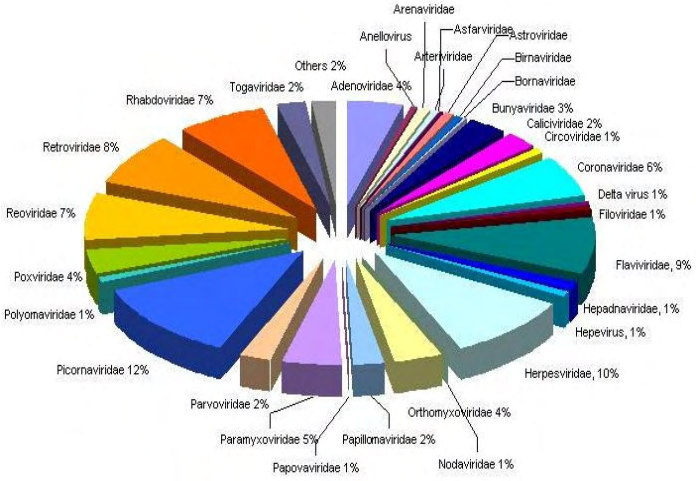

Fig. 1. Defra Biochip version 4.1, percentage of oligonucleotides representing 29 virus families.

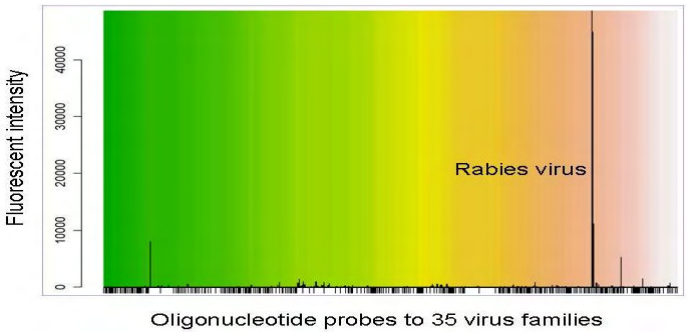

Fig. 2. Detection of rabies virus in the brain of a patient from case of travel acquired rabies in Belfast, Ireland

Table 1. Detection of equine encephalosis virus in febrile horses. The microarray data is normalized against a control slide and values are sorted on the average.

Virus name	Probes	t value	p value	Average
Equine encephalosis virus	9	3.07	0.0076	5.7
Pongine herpesvirus 4	6	2.79	0.0191	3.14
Gallid herpesvirus 3	23	2.46	0.011	1.65
Human herpesvirus 7	23	3.16	0.0022	1.12

Discussion and conclusions
Our results have provided evidence on the potential of microarray in investigation of viral diseases in human and animals. The microarray successfully identified the viral agents in clinical samples obtained from cases of farmed and feral animal and human disease.

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NEW REAL-TIME PCR USING UNIVERSAL PROBE LIBRARY (UPL) FOR DETECTION OF AFRICAN SWINE FEVER VIRUS (ASFV)

Jovita FERNÁNDEZ-PINERO, Carmina GALLARDO, Ana ROBLES, Covadonga PÉREZ, Concepción GÓMEZ, Raquel NIETO, Marisa ARIAS

EU Reference Laboratory for African Swine Fever, Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, Madrid, Spain

Keywords: African swine fever, real-time PCR, UPL, molecular diagnosis

Introduction and objectives

African Swine Fever (ASF) is a highly contagious infectious disease affecting *Suidae*, caused by a complex DNA virus of the *Asfarviridae*. It is a notifiable disease, endemic in large parts of Sub-Saharan Africa and in Sardinia, producing great economic losses. In 2007, ASF emerged in the Caucasus for the first time, and has since spread to several countries in the region. Molecular diagnosis of ASF relies on a limited number of conventional and real-time PCR methods[1, 3-5].

Universal Probe Library (UPL), recently commercialized by *Roche Applied Science*, is a collection of short hydrolysis LNA probes, originally designed for gene expression analysis and offered as a universal detection system. They are 8-9 LNA residues in length, labelled with FAM at the 5' and a dark quencher dye at the 3'. The main advantages of UPL probes for their application in the infectious diseases diagnosis are the reasonable low cost, the short time of delivery, and the ready-to-use presentation. The combination of a specific primer set and an appropriate UPL probe will allow specific and sensitive detection of ASF virus (ASFV) by real-time PCR at a comparably lower cost. This work presents the development and simple detection of ASFV by real-time PCR employing this new approach. Laboratories, mainly those from countries where ASF is circulating, could benefit from the simple way to acquire the UPL probes.

Materials and methods

ASFV specific primer set was designed and corresponding UPL probe was selected from conserved VP72 genome region with the aid of specific *ProbeFinder Software (Roche Applied Science),* and were further checked for ASFV specificity using *Blast* tool. Additionally, a conventional TaqMan probe was designed within the same region, to be combined with the same selected primer set, for comparison of probe efficacy and convenience.

A collection of 14 ASFV isolates from different origin (Africa, Europe, and America) was employed in the study. Classical swine fever virus (CSFV) and other porcine viruses were tested in specificity assays. Clinical material collected from ASFV experimentally infected pigs was also used in the study. Total nucleic acids were extracted from samples using *High Pure PCR Template Preparation Kit (Roche)*, following manufacturer's instructions.

Real-time PCR protocols were optimised using *LightCycler 480 Probes Master kit (Roche Applied Science).* Established ASF real-time PCR[2] *(QuantiFast Probe PCR kit, Qiagen)* using primers and probe described previously[3] was performed as reference method.

Results

First real-time PCR assays were carried out to evaluate the competence of the selected UPL probe for the detection of ASFV. All 14 ASFV isolates analysed were properly detected by using the novel UPL real-time PCR, showing very similar Ct values than those obtained by the real-time PCR using the TaqMan probe designed to be combined with the same primer set. In the same way, higher Ct values (ranging from 1 to 5 cycles) were obtained using the protocol of the reference PCR[2,3] established in the lab.

Sensitivity assays using duplicates of serial dilutions of ASFV Kenya 07, Sardinia 88, and Cape Verde 97 isolates showed that UPL PCR gives a detection limit quite similar to that provided by designed TaqMan PCR, while it is 10-fold more sensitive than the established reference real-time method[2,3].

The analysis of EDTA-blood and serum samples collected daily from a pig, experimentally infected with 10^5 $HADU_{50}$ of ASFV Spain70 strain, showed positive amplification from 1st and 3rd day post-inoculation (dpi), respectively, by using developed UPL or TaqMan PCR methods.

Specificity of the novel UPL PCR was further evaluated using a collection of clinical samples from experimentally ASFV infected and non-infected pigs, as well as testing CSFV, and other viruses affecting swine. Fluorescence signal was observed exclusively in ASFV infected samples.

Discussion and conclusions

Some real-time PCR methods have been already developed or are under development for the detection of pathogens, such as human immunodeficiency virus (HIV), using *Universal Probe Library (UPL)* probes (personal communication). This work describes for the first time a real-time PCR method for the detection of a virus affecting animals using this technology. The method employs an ASFV specific primer set and an appropriate UPL probe that assures a specific amplification result. First studies prove that the developed UPL real-time PCR is a sensitive and specific method to detect a wide range of ASFV isolates and a useful tool in clinical specimens.

Additionally, assays to select an internal control using UPL probes to be included in a duplex PCR format are ongoing.

Although validation experiments are still required, the first results show the developed UPL PCR method as a promising tool for rapid and affordable molecular diagnosis of ASF. Moreover, the convenience of UPL probes in routine diagnostic laboratories is increased by the low cost, the simplicity to order and use, and the compatibility with any real-time PCR instrument and reagents.

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A SENSITIVE ONE-STEP REAL-TIME PCR TECHNOLOGY USED IN VETERINARY VIROLOGY AND DISEASE CONTROL

Szabolcs KÖKÉNY

Applied Biosystems Hungary Ltd, Budapest, Hungary

Real-time, reverse transcription polymerase chain reaction (rRT-PCR) has become one of the most widely used methods in the field of molecular diagnostics and research.

Inspired by Veterinary Laboratory Diagnosticians, Applied Biosystems develops, manufacturers and delivers comprehensive next generation molecular testing tools and services that instill confidence in the results obtained from monitoring and surveillance programs. Applied Biosystems and the VLA announced a strategic collaboration to manufacture and commercialize the Newcastle disease environmental detection kits. This collaboration is intended to help early detection of these harmful bird diseases, which is a critical step in managing the threat posed by these diseases.

The Animal Health Solutions from Applied Biosystems, the Real-Time PCR and workflow solution tools provide a larger window of detection, works with diverse sample matrices (blood, milk, serum, plasma, tissue), generates results with higher sensitivity and greater specificity, and reduces false positive and negatives, minimizes cross reaction and contamination issues and allows for less invasive sample collection. Integration of these assays from manual RNA isolation into automated sample preparation platforms for nucleic acid extraction increases the rate and Real-time viral detection *in vivo* provides sufficient information regarding multiple steps in infection and disease progress, which will be valuable for the prevention and control of viral infection. These assays can be further enhanced by using internal controls to validate test results. We will discuss the complexities of integrating and using this information to understand the portfolio of Applied Biosystems in Animal Health Initiative as well as highlight the potential for diagnostic and other forms of biological analysis.

RECOMBINANT NS3 AND MONOCLONAL ANTIBODIES FOR SERODIAGNOSIS OF PESTIVIRUSES INFECTION BY COMPETITIVE ELISA

Giulia PEZZONI, Lidia STERCOLI, Paolo CORDIOLI and Emiliana BROCCHI

Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna, Brescia Italy

Keywords: monoclonal antibodies, Pestiviruses, recombinant NS3, serology

Introduction and objectives

Bovine Viral Diarrhea Virus (BVDV) belongs to Pestivirus group with viruses responsible for Border Disease and Classical Swine Fever (CSF); the three viruses are antigenically related. Currently, serological assays for Pestiviruses are based on the detection of antibodies against the immunogenic and conserved non structural protein 3 (NS3). The objective of the present work was the development of a functional antibody-detection competitive ELISA for pestiviruses using the recombinant antigen NS3 expressed in a baculovirus-system associated with monoclonal antibodies (MAbs), in order to improve the standardization and performance of the serological test.

Materials and methods

Recombinant NS3E. The NTPase-elicase domain of NS3 was expressed in a baculovirus system as described by Pezzoni et al. and used as crude cell lysate.

Indirect-trapping ELISA. An home-made indirect ELISA, routinely used in the laboratory to detect anti-BVDV antibodies in cattle sera, was used for comparison with the newly developed competitive ELISA. The test is performed with BVDV antigen trapped by the anti-NS3 MAb 3H4, sera are examined at 1/50 dilution and the presence of specific antibody bound to the antigen is measured with anti-bovine IgG MAbs peroxidase-conjugated.

NS3-competitive ELISA. The new competitive ELISA for the detection of anti-pestivirus antibody was substantially similar to the previously described test based on MAbs and BVDV antigen (Brocchi et al.). Briefly, the MAb 3H4 was adsorbed onto NUNC maxisorp microplate at a saturating concentration to trap the recombinant NS3. Sera diluted 1/4 and a pre-determined, optimal dilution of the selected MAb 3A3, peroxidase-conjugated, were sequentially incubated. After the development of the reaction, results were expressed as percentage of competition generated by sera with respect to the non-inhibited reaction between the immune-captured antigen and the labelled MAb.

CSFV-competitive ELISA. A competitive ELISA for CSFV-specific antibodies, provided by the National Reference Laboratory (IZS Perugia), was used as confirmatory test.

Sera. 414 bovine and 2821 swine field sera were examined in parallel against BVD viral antigen and the recombinant NS3 in trapping and/or competitive ELISAs. In addition, sequential sera from 11 pigs experimentally vaccinated with the CSF China strain and subsequently challenged with a pathogenic virus were tested in parallel by competitive ELISAs for antibody detection to NS3 and to CSFV.

Results

Results of testing 414 bovine sera against the native and the recombinant NS3 antigen in indirect-trapping ELISA showed a concordance of 100%.

The NS3-competitive ELISA was evaluated on 369 out of 414 bovine field sera classified as positive and negative according their reactivity with the viral antigen in trapping ELISA. The frequency distribution of percentages of competition obtained showed a clear separation between positive and negative sera (fig.1), and the cut-off was set at 60% competition. The two ELISAs showed 100% concordance, with 147 samples positive and 222 negative in both tests.

The NS3-competitive ELISA was also applied to test 2821 swine field sera classified as positive and negative in the competitive ELISA based on same MAbs but on BVDV as source of antigen. With a cut-off set at 60% competition in the NS3-ELISA, the concordance between the two tests was 99.1%, with 319 positive and 2479 negative sample in both tests; only 23 sera (0.8%) provided discordant results, 8 of them with borderline values (figure 1). Antibodies in positive pig sera were elicited by infection with pestiviruses other then CSFV, as none of them reacted in the CSFV-specific ELISA.

The capability of NS3-competitive ELISA to recognize also immune response elicited by CSF virus was investigated using a panel of experimental sera: the kinetic of the humoral response detected by the recombinant ELISA consistently correlated with results provided by the CSFV-specific ELISA (figure 2). The evaluation of end-point titers showed evidence of antibody booster 15 days post–challenge.

Discussion and conclusions

This study reports on the use of a baculovirus-expressed NS3 as source of antigen in two ELISA formats (competitive and trapping) for detection of anti-pestiviruses antibodies. Results provided evidence that the recombinant antigen can successfully substitute the virus in serological diagnosis of infection caused by pestiviruses; the competitive ELISA is preferable since it is equivalently applicable to any animal species. The use of the recombinant antigen in association with characterized MAbs makes the production and yield of biological reagents safer and easier and ensures improved standardization and reproducibility of serological tests.

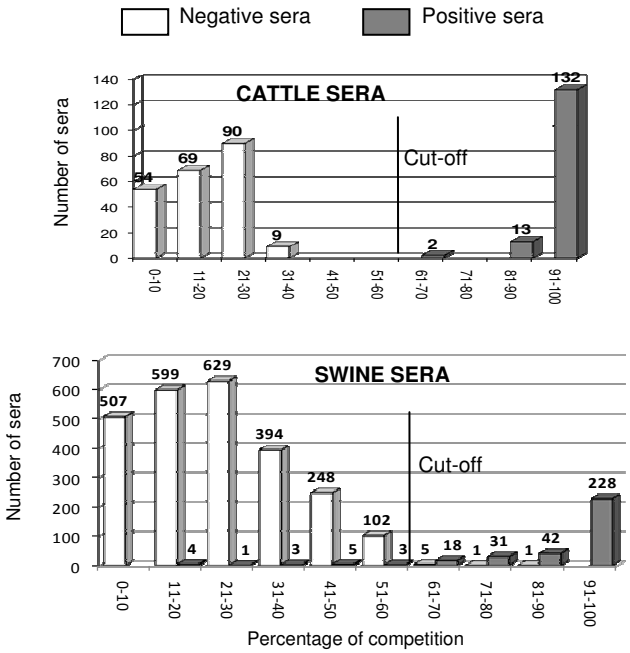

Figure 1. % of competition in NS3 ELISA of bovine (n. 369) and pig (n. 2821) sera, classified as positive or negative based on their reactivity with BVD viral antigen in trapping or competitive ELISA respectively

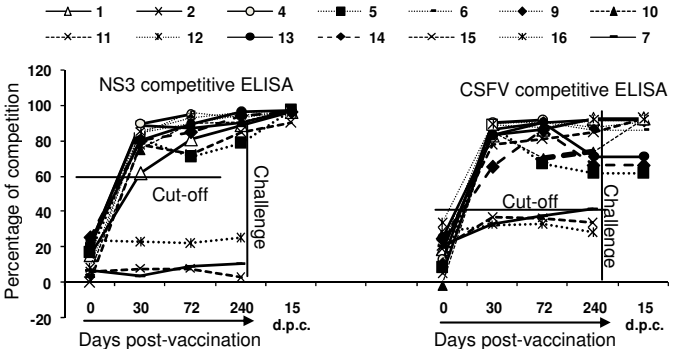

Figure 2. Comparative results of NS3 and CSFV ELISAs for testing pigs experimentally immunised against CSFV (7,15 and 16 are control pigs)

Acknowledgements

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RT-PCR DETECTION AND PHYLOGENETIC ANALYSIS OF HUNGARIAN EQUINE INFECTIOUS ANAEMIA VIRUS STRAINS

Miklós RUSVAI[1], Tamás BAKONYI[2,3], Ákos HORNYÁK[2], Gyula BALKA[1], Aymeric HANS[4], Norbert NOWOTNY[3]

[1]*Department of Pathology and Forensic Veterinary Medicine, Faculty of Veterinary Science, Szent István University, Budapest, Hungary*
[2]*Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Budapest, Hungary*
[3]*Zoonoses and Emerging Infections Group, Clinical Virology, Department of Pathobiology, University of Veterinary Medicine, Vienna, Austria*
[4]*Laboratory for Studies and Research in Equine Diseases, AFSSA, Douzlé, France*

Keywords: ELISA, equine infectious anaemia virus, phylogenetic analysis, RT-PCR

Introduction and objectives

Equine infectious anaemia (EIA) is a slowly developing, destructive disease of equids ([1]), caused by the Equine infectious anaemia virus (EIAV, *Retroviridae, Lentivirus*). Due to the lethal character of the disease, and the lack of vaccine protection, in most countries EIA is a communicable disease, and infected horses are exterminated. The horses are usually serologically screened with agar-gel immunodiffusion test (Coggins test, [2]). In Hungary, the serological investigation of horses in every three years is obligatory. Within the last two decades only sporadic and predominantly imported cases were found.

In this presentation we show the results of two surveys on EIAV infections in Hungary in 2008 and in 2009.

Materials and methods

In 2008 and 2009 sera were collected from 1425 and 1501 horses (respectively), which were kept on the Western and Southern part of Hungary. The sera were tested for EIAV antibodies by competitive ELISA (Idexx). Positive results were retested by using the Coggins test two times. Horses, which were positive in both the ELISA and the repeated Coggins tests, were exterminated. Peripheral blood mononuclear cells (PBMCs) were collected from two horses (№ Hu 109-09 and Hu 110-09) prior to euthanasia. Four further horses found positive by the Veterinary Diagnostic Directorate of the Central Agricultural Office (№ Hu 267-09, Hu 268-09, Hu 273-09 and Hu 274-09) were exterminated, tissue samples (spleen, liver, heart, kidneys) and PBMCs were collected.

A reverse transcription - polymerase chain reaction (RT-PCR) assay has been developed to detect EIAV nucleic acid in the samples. Primers anneal the most conserved genomic region (5' UTR - gag) of the virus, between nt positions 198 and 504 of the EIAVuk clone (AF016316). As positive control, an EIAV infected spleen sample from France (№ Fr 1980-09) kindly provided by Prof. Marc Eloit was used.

After RT-PCR the amplification products were subjected to direct sequencing, sequences were identified by BLAST search, and were aligned with all available EIAV sequences of the corresponding genomic region. A neighbour-joining (NJ) tree was constructed to illustrate he genetic relationships between the strains.

Results

In 2008 antibodies against EIAV were found in 6 sera by the competitive ELISA, and in 2 cases the repeated Coggins tests also confirmed the results. In 2009 the 3 ELISA positive blood samples were negative in the Coggins tests.

EIAV-specific nucleic acid sequences were detected in five Hungarian horses' samples by the RT-PCR assay. The NJ tree of the sequences determined in this study, and sequences from the GenBank database is shown in Figure 1. Several sequences deposited by other groups previously (AF016313, AB008196, AF033820, AF028231, AF028232, U01866, M16757, and M87581) were identical in this region; therefore they are represented with only one sequence (AF033820) on the NJ tree.

The identified sequences cluster into three main groups. One group is formed by EIAV sequences from two horses of a Hungarian stable, together with a sequence from Japan, and several sequences of unknown origins (represented by AF033820). One Hungarian EIAV and the sequence from the French sample, which was used as reference, clusters together with two Chinese EIAV strains, one of them is indicated as an attenuated vaccine strain (AF327878). The third cluster is formed by another two sequences from horses of one Hungarian stable, and one EIAV strain from Italy. Sequences, obtained from the same populations differ from each other in a few nucleotides.

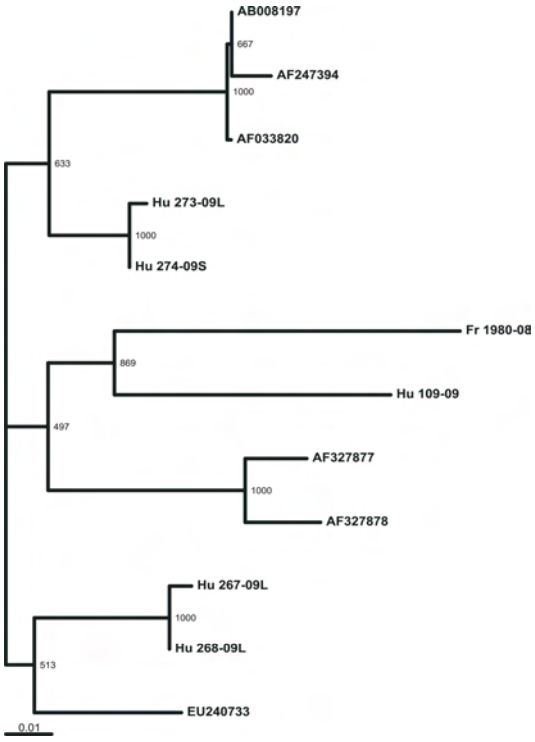

Figure 1.: NJ tree representing the genetic relationships between EIAV sequences. Sequences derived from the GenBank database are labelled by accession numbers. L. lymphocyte sample, S: spleen sample. Internal labels show the bootstrap values of 1000 replicates. Bar represents the genetic distance.

Discussions and conclusions

The ELISA test applied in the survey is more sensitive then the Coggins test; hence the results must be taken into consideration even when the Coggins test is negative. EIAV infection is for life, horses once infected will never get rid of the virus. The level of seropositivity is increasing with time, after recurring episodes of viremia, therefore ELISA positivity sometimes precedes Coggins positivity even with years. The EIAV infected horses represent hazards for solipeds in the area, since blood sucking ectoparasites (horseflies, mosquitoes etc.) may transfer contaminated blood from one animal to another. The developed RT-PCR recognizes the different genotypes of EIAV, and may serve as an additional tool to identify infected horses in case of contradictory results.

Acknowledgements

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VALIDATION OF NOVEL REAL-TIME PCRS FOR THE SIMULTANEOUS DIAGNOSIS OF THREE DIFFERENT VIRAL ENCEPHALOMYELITIS INFECTIONS OF HORSES

Marcello SALA[1], Antonella CERSINI[1], Armando DAMIANI[2], Maria Teresa SCICLUNA[1], Giuseppe MANNA[1], Valentina SPALLUCCI[1], Andrea CAPRIOLI[1], Maria Ilaria CIABATTI[1], Gian Luca AUTORINO[1]

[1]Istituto Zooprofilattico Sperimentale Lazio e Toscana, Roma, Italy; [2]Department of Veterinary Microbiology and Immunology, Cornell University,USA

Keywords: Borna disease virus, Equine herpesvirus 1, Real-Time PCR Validation, West Nile virus

Introduction and objectives

This study describes the validation of novel Real-time PCRs for the simultaneous diagnosis of viral encephalomyelitis infections representing a potential risk of diffusion in the Italian and European horse population. The methods used are based on the selection of highly conserved molecular targets, previously not described, from the genomes of Equine Herpesvirus type 1 (EHV-1, gene gE), of West Nile virus (WNV, gene NS1-NS2) and of Borna Disease virus (BDV, gene M). The three methods had already been verified and resulted in having high analytical sensitivity and specificity (1,2,3). The aim of this study was to provide validated virological methods for the simultaneous and differential diagnosis to be employed by the Italian Veterinary Laboratory Network during the surveillance of these infections.

Materials and methods

For the validation of the methods, three series of 20 blind samples, consisting of horse brain tissue, positive or negative for the three viruses were prepared and sent to 15 Italian and to 2 foreign Laboratories. Each series consisted of 10 negative samples, three positive for WNV, three positive for BDV and four positive for EHV-1: every sample was coded uniquely. The negative samples were obtained from the spinal chord of a slaughtered horse resulting free for the three viruses. The same spinal chord was seeded positive with the reference strains of the viruses (EHV –1 Army 83, WND Egypt 101 and BDV H1766). These samples were confirmed for their specific positivity and absence of possible cross-contamination. The participants were asked to examine each panel for the three infections in three independent sessions using the procedures provided by the organizer. For each Laboratory, the following parameters were evaluated: sensitivity, specificity and accuracy of the overall results. The repeatability (intra-laboratory) and the reproducibility (inter-laboratory) of the individual (i.e. separately for WNV, EHV-1 and BDV) and overall results for the three viruses, were assessed for the participants. Cohen's Kappa statistic was used for the calculation of the chance-corrected probability of agreement between the reference values (true status of the samples) and the results provided for the same samples by the single and by all the 17 Laboratories (4). The degree of agreement beyond chance was determined using the Landis-Kock Scale for the interpretation of Kappa values ranging from 0 (no better than chance) to 1 (perfect agreement).The values of these parameters were considered satisfactory if accuracy was equal to 100% and Kappa >0.8.

Results

The validation of the methods was performed considering the 60 results provided by each Laboratory in the three independent sessions (20x3) for the intra-laboratory evaluation and the overall results (N=1020) for the inter-laboratory evaluation. Fifteen of the seventeen participants obtained an accuracy equal to 100% and Kappa equal to 1 with respect to the reference value for the single diagnosis as also for the overall results for the three viruses. These Laboratories showed 100% sensitivity and specificity and were in complete agreement with the expected results, giving a perfect repeatability. Using only the results provided by these Laboratories (N results=900), the inter-laboratory evaluation also provided a perfect reproducibility (Kappa=1) and a 100% accuracy. The remaining two Laboratories obtained unsatisfactory results. In particular, Laboratory N°10 misclassified a total of 6 samples for WNV (3 false positive and 3 false negative) giving a 67% sensitivity, a 94% specificity and a 90% accuracy, producing a moderate agreement with the reference values (kappa=0.60). The same Laboratory also misclassified a total of 4 samples for EHV-1 (2 false positive and 2 false negative) giving an 83% sensitivity, a 96% specificity, a 93% accuracy and a Kappa=0,79. Considering the overall results for the three viruses, Laboratory N°10 produced an unsatifactory sensitivity (83%), specificity (87%), accuracy (85%) and an incomplete agreement beyond chance with respect to the reference values (Kappa=0.70). Laboratory N°12 misclassified a total of 8 sample for EHV-1 (4 false positive and 4 false negative) giving a 67% sensitivity, a 92% specificity and an 87% accuracy, resulting in a moderate agreement with the reference values (kappa=0.58). Considering the overall results for the three viruses, Laboratory N°12 obtained an unsatisfactory sensitivity (87%), specificity (90%), accuracy (88%) and an incomplete agreemet beyond chance with respect to the reference values (Kappa=0.76).

These results demonstrated that all participating Laboratories were accurate and precise for the diagnosis of BDV, while 2 of them gave unsatisfactory sensitivity, specificity, accuracy and precision for EHV-1 and only one Laboratory was also unsatisfactory for WNV. Such discordant results could be imputed to systematic errors.

Notwithstanding the results of the two inaccurate Laboratories, a high level of reproducibility (Multiple Kappa >0.96) and an acceptable accuracy (>98%) were obtained for the trial when including all the Laboratories (Table 1).

Discussion and conclusions

The results obtained for the parameters evaluated for the validation of the Real-time PCRs expressed a high sensitivity and specificity for each of the three methods which were employed for the simultaneous detection of BDV, EHV-1 and WNV genomes. In view of the results obtained, all the methods satisfy the criteria set for the validation for the polymerase chain reaction methods used for the diagnosis of infectious diseases prescribed by OIE (5). Routine diagnostic results which are available since 2008 are contributing to the on-going field validation of the methods. This continuous validation process constitutes a fundamental prerequisite of these specific methods for their use in the simultaneous diagnosis of such viral infections, supportive of surveillance programmes. The validated methods, made available to the Italian Official Laboratory Network, will allow an improvement of the diagnostic effectiveness, ensuring comparable results throughout.

Acknowledgements

The Italian Network of Official Laboratories, the Italian Research Veterinary Istitutions, the CRL for equine diseases (AFSSA – Maison Alfort) and the Duncombe Laboratory (Caen – France). This work was part of a research project financed by the Italian Ministry of Health.

Table 1: Summary of the PCRs Validation outcome: (R1=repeatability; R2=Reproducibility; K= Kappa; a=accuracy)

	ID. Laboratory	WNV		BDV		EHV-1		In parallel	
		K	a	K	a	K	a	K	a
R1	N°10	***0,60***	***90%***	1	100%	***0,79***	***93%***	***0,70***	***85%***
	N°12	1	100%	1	100%	***0,58***	***87%***	***0,77***	***88%***
	Each of the remaining 15 Labs	1	100%	1	100%	1	100%	1	100%
R2	*15 accurate Labs (N=900)	1	100%	1	100%	1	100%	1	100%
	17 Labs (N=1020)	0,97	99,4%	0,99	99,9%	0,96	98.8%	0,96	98,3%

*** accurate and precise labs**

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IN SITU PROXIMITY LIGATION ASSAY FOR STUDYING VIRUS AND PATHOGENESIS

Jonas J. WENSMAN[1], Karl-Johan LEUCHOWIUS[2], Anna-Lena BERG[3], Liv BODE[4], Hanns LUDWIG[5], Sándor BELÁK[1,6], Ulf LANDEGREN[2], Ola SÖDERBERG[2], Mikael BERG[1]

[1]Swedish University of Agricultural Sciences, Department of Biomedical Sciences and Veterinary Public Health, Uppsala, Sweden; [2]Uppsala University, Department of Genetics and Pathology, Uppsala, Sweden; [3]Astrazeneca R&D, Safety Assessment, Södertälje, Sweden; [4]Robert Koch Institute, Berlin, Germany; [5]Freie Universität Berlin, Institute of Virology, Berlin, Germany; [6]National Veterinary Institute, Department of Virology, Immunobiology and Parasitology, Uppsala, Sweden

Keywords: in situ proximity ligation assay, protein interactions, virus detection

Introduction and objectives

In situ proximity ligation assay (isPLA) is a novel method for studying proteins and protein-protein interactions in cells or tissues (3). The principles of isPLA are shown in Figure 1. Both primary and secondary antibodies can be oligonucleotide-conjugated and used as proximity probes.

As a model for virus detection by isPLA, Borna Disease Virus (BDV) was used. BDV is a neurotropic RNA-virus and the causative agent of neurological disorders in a wide range of animals, possibly including human. In some animals like cats, the viral load is low, and hence there is a need for more sensitive detection methods, such as isPLA (2). Beside virus detection, isPLA has a great potential to give further knowledge in pathogenetic mechanisms, by studying host-virus protein interactions.

Materials and methods

C6 (rat astrocytoma) cells, persistently infected with BDV or non-infected, were used in the initial evaluation of the BDV isPLA. Formalin-fixed paraffin-embedded brain tissue from experimentally and naturally BDV-infected animals was used, as well as non-infected animals as negative controls. A rabbit polyclonal and a murine monoclonal anti-BDVp23 antibody were used as primary antibodies. As secondary antibodies, oligonucleotide-conjugated anti-rabbit and anti-mouse antibodies were used (Duolink in situ PLA, Olink Biosciences, Uppsala, Sweden). Ligation, rolling circle amplification (RCA) and hybridisation of fluorescently labelled oligonucleotides were performed according to the manufacturer's instructions (Duolink in situ PLA). The fluorescence signals were converted into bright field microscopy signals using Dako DuoCISH (Dako A/S, Glostrup, Denmark).

Results

The isPLA for BDV detection was initially evaluated in infected and non-infected cells. There were no background in the non-infected cells, while there were intensive signal in the BDV-infected cells. In brain tissue from experimentally infected rat, there was a very intense signal compared to the negative control. The same was seen in a German horse with classical Borna disease (BD). Further, cats with staggering disease (also known as feline BD) were positive in the BDV isPLA.

Discussion and conclusions

In situ PLA is a powerful tool for detection of proteins and protein-protein interactions. In this study, isPLA was used to detect BDV, a neurotropic RNA-virus infecting a wide range of animals. There is an increasing knowledge of host-virus protein interactions in BDV infection, although most are only shown *in vitro* or in experimental infections (1). Ideally, the pathogenesis should be studied in natural infection, if possible. We believe that isPLA could provide such a possibility. By using antibodies towards host and virus proteins to study possible interactions, more insight into the mechanisms of disease would be provided.

Acknowledgements

The authors would like to thank professor G. Gosztonyi, Dr. F. Södersten and Dr. G. Hestvik for kindly providing brain tissue material and mrs Å. Gessbo for technical assistance. This work was supported by grants from Swedish Foundation for Equine Research.

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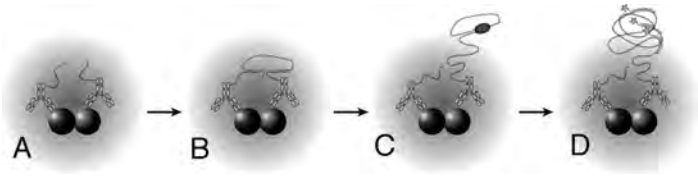

Figure 1. A pair of oligonucleotide-conjugated antibodies (proximity probes) are used to probe the target protein(s). If the target proteins are in proximity (A), the two oligonucleotide arms will be brought together by binding of the antibodies, and can be used as a ligation template for the creation of a DNA circle (B). The DNA circle can then be used to extend one of the oligonucleotide arms through RCA (C). Individual replication products fold into bundles of DNA that can be visualized by fluorescently labelled detection oligonucleotides (D).

DEVELOPMENT OF MICROSPHERE-BASED IMMUNOASSAY FOR DETECTION OF ANTIBODIES TO BOVINE VIRAL DIARRHEA VIRUS

Hongyan XIA[1], Lihong LIU[2], István KISS[2], Ann NORDENGRAHN[3], Ronnie ERIKSSON[4], Jonas BLOMBERG[4], Malik MERZA[3], Sándor BELÁK[1, 2]

[1]Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, SE-751 89 Uppsala, Sweden;
[2]Joint R&D Division in Virology, The National Veterinary Institute & The Swedish University of Agricultural Sciences, SE-751 89 Uppsala, Sweden;
[3]Svanova Biotech AB, Uppsala Science Park, Glunten, S-751 83 Uppsala, Sweden; 4Laboratory Section of Virology, Department of Medical Sciences, SE- 751 85 Uppsala, Sweden

Introduction and objectives

Bovine viral diarrhoea virus genotype 1 (BVDV-1), genotype 2 (BVDV-2) and genotype 3 (BVDV-3) belongs to the genus Pestivirus of the family Flaviviridae. The BVDV infection is distributed worldwide and cause important financial lost to the livestock industry. The prevalence of the infection could reach a level of 1-2% cattle persistently infected (PI) and 60-85% of antibody-positive cattle (1). Currently, ELISAs are widely used for mass screening and to monitor the infection status of individual animals and herds. Recent developments in microsphere-based flow cytometric assays (the best-established microsphere assay system is the xMAP system from Luminex Corp) make it possible to adapt the ELISA from solid phase to liquid phase (microsphere immunoassay (MIA)), thereby not only provide multiplex capacity, but also leading to high sensitivity and specificity and reproducibility compared to ELISA. In our study, we aim to develop serological assay to detect antibodies to BVDV by Luminex technology and to compare the performance of the assay with ELISA.

Materials and methods

Monoclonal antibodies (mAbs) was obtained from Svanova Biotech AB (Uppsala, Sweden). Microspheres were purchased from Luminex Corp (Austin, TX). Coupling reagents and Biotinylation reagents were from Pierce. Serum samples were collected from Sweden. Coupling capture mAbs to microspheres was performed according to the instructions from Luminex. Biotinylation of mAbs was done according to the instruction from Pierce Biotechnology (Rockford, IL). A blocking immunoassay was performed to detect antibodies in serum. In this assay, the mAbs coupled to microspheres was incubated with viral antigen and serum for 30 min, followed by incubating with biotinylated mAbs. Specific reactions were detected by a reporter fluorochrome (R-phycoerythrin) conjugated streptavidin. The median fluorescence intensity (MFI) was recorded on Luminex 200 system.

Results

Efficiency of coupling of mAbs to the microspheres was different. The one with higher coupling efficiency was selected as capture antibody. The optimal amount of protein coupled to the 1 million microspheres was 2 µg. The antibody-conjugated microspheres are stable more than 10 months at 4℃. Serum dilution at 1:2 gave highest sensitivity and specificity. The sensitivity and specificity of the assay were comparable to ELISA. Moreover, by using suspension microspheres, the incubation time could be reduced to 30 min and no wash procedures were required. This greatly shortens the detection time. In conclusion, the suspension immunoassay described here is sensitive and specific assay for fast detection of Abs against BVDV.

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DEVELOPMENT OF AN RT-PCR TEST FOR CHICKEN ASTROVIRUSES

Victoria J. SMYTH[1], Heather L. JEWHURST[1], Brian M. ADAIR[2], Daniel TODD[2]

[1]Queen's University, Agri-Food and Biosciences Institute, Stormont, Belfast , BT4 3SD, Northern Ireland;
[2]Agri-Food and Biosciences Institute, Stormont, Belfast , BT4 3SD, Northern Ireland.

Keywords: astrovirus, chicken, detection, RT-PCR

Introduction and objectives

Astroviruses are small spherical enteric viruses measuring 25 to 30 nm in diameter with a characteristic 5- or 6 -pointed star-like appearance by EM. However, this star-like appearance is generally not apparent so other means of identification are necessary to distinguish them from other small round viruses of similar size. Chicken astrovirus (CAstV) infections are associated with growth depression in chicks, which we have demonstrated experimentally in our laboratory [3] and may contribute to runting stunting syndrome. The only CAstV to be characterised to date was reported in 2004 [1] but the sequence was not revealed. Like other astroviruses, CAstV has a single-stranded RNA genome with a polyA tail. There is one published RT-PCR test specifically for detecting CAstV, which was developed from partial polymerase *(pol*) gene sequences from US CAstVs, that are similar to the 2004 CAstV (refCAstV). We have recently discovered novel CAstVs [4], which are antigenically different from the refCAstV, and which the existing CAstV RT-PCR test [2] does not detect due to changes in the RNA sequence. Sequencing of partial *pol* regions from multiple CAstV field samples and isolates has allowed us to identify regions of the highest conservation in which to site an RT-PCR test. The objective of this work was to develop an RT-PCR test that was CAstV specific and which would detect as many different variants as possible including novel CAstVs and to apply the assay to testing of field and longitudinal survey samples.

Materials and methods

Six isolates of CAstV from UK, Ireland, South Africa and the Netherlands, which were grown in eggs or cell cultures, were processed as previously described [3]. Samples of faeces, gut contents or faecal swabs were obtained from sick chickens collected from UK and German broiler flocks from 9 different locations and 5 poultry producer organisations, that were experiencing growth depression problems between 2004 and 2008. Homogenates (10%) were clarified by centrifugation at 3,000g for 20 min and RNA extracted from the supernatants and from the passaged isolates. These RNAs were subjected to one step RT-PCR using a 4-base degenerate forward primer based in the mid-*pol* area and a non-degenerate reverse primer located in a highly conserved region at the 3` end of the *pol* ORF, which produced a 510 bp amplicon. A selection of field sample amplicons were sequenced to ensure specificity and to determine their CAstV type. The specificity of the assay was tested against avian nephritis virus (ANV) and duck hepatitis viruses (DHV)-2 and -3, which are also astroviruses. The sensitivity of the test was estimated from a 10-fold dilution series of *in vitro* transcribed RNA of known concentration from a cloned 858 bp CAstV amplicon. The assay was applied to 52 field samples and longitudinal survey samples from 4 flocks, that were later shown to display below average performances. Gut contents were taken from 12 birds at 10 timepoints ranging from 0 to 42 days. Each set of 12 samples was grouped into 4 pools and processed as above.

Results

Assay Specificity and Sensitivity. The assay detected CAstV in all six CAstV isolates, whereas the published test was negative for half of these. The new assay was negative for the ANV, DHV-2 and DHV-3 isolates. The limit of detection for the test was 60 copies using a 10-fold dilution series of *in vitro* RNA transcripts.

Field Samples. The assay positively detected CAstV in 96% (50/52) of UK and German field samples from problem flocks, whereas the published test detected only 58% of the same samples. Amplicons from 14 of the field samples and the 6 isolates were sequenced and found to comprise 70% Group I and 30% Group II CAstVs, Fig 1. The two groups were confirmed by phylogenetic analysis. Viruses in the novel group (II) exhibited a high nucleotide (nt) similarity (>94%) but shared only 76-79% nt identity with Group I CAstVs. Group I CAstVs shared 85-99% nt identity.

Longitudinal Survey. 67.5% of gut contents were positive for CAstV by this test across all timepoints except day 0 which were all CAstV negative. Highest CAstV detection levels across all flocks occurred in samples at days 4 or 5 (14/16; 87.5%), day 7 (12/16; 75%) and day 35 (12/16; 75%). Flocks A and C were CAstV-negative at half of the timepoints, with CAstV being detected in 33.3% and 30.0% of the samples respectively. With flocks B and D, CAstVs were detected at more timepoints (flock B, 8; flock D, 9) and in higher numbers of pooled samples (flock B, 72.5%; flock D, 67.5%) and these were also the two worst performing flocks of the four.

Discussion

The availability of a sensitive CAstV RT-PCR test provides a useful diagnostic tool with which to begin investigations into the pathogenicity and epidemiology of CAstV infections. This new test has wider scope than the previous test which fails to detect many CAstV sequences due to the high sequence diversity exhibited by RNA viruses and because it was designed on limited sequence data. Group I CAstVs were more commonly detected than Group II CAstVs due to either higher prevalence or preferential amplification. A longitudinal survey of 4 flocks in NI has shown that CAstV infections are endemic and their relevance to growth depression can now be investigated.

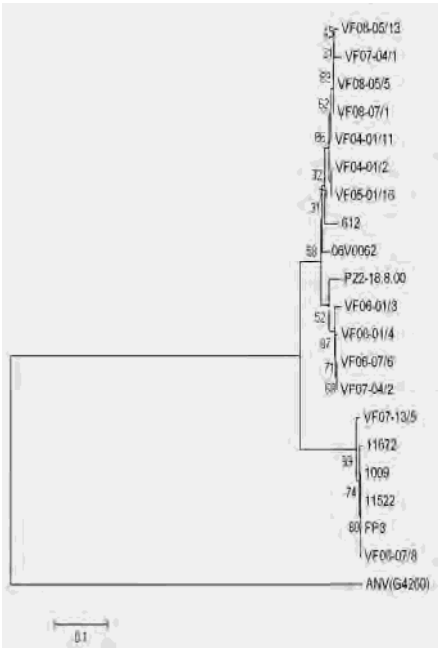

Fig 1. CAstV nt partial pol neighbour joining tree, rooted by ANV. Upper group (14 sequences), Group I; lower group (6 sequences), Group II.

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DETECTION AND PARTIAL CHARACTERIZATION OF PARAMYXOVIRUSES FROM REPTILES

Rachel E. MARSCHANG[1], Maha D.A. SHUKUR[1], Michael PEES[2], Volker SCHMIDT[2], Jürgen SEYBOLD[3], Jens W. FROST[4], Silvia BLAHAK[5], Tibor PAPP[1]

[1]*Institut für Umwelt und Tierhygiene, Universität Hohenheim, Stuttgart, Germany;*
[2]*Klinik für Vögel und Reptilien, Leipzig University, Leipzig, Germany;*
[3]*Kleintierzentrum Mitteltal, Max-Eyth-Str. 36, 72270 Baierbronn-Mitteltal, Germany*
[4]*Landesbetrieb Hessisches Landeslabor, Giessen, Germany;*
[5]*Staatliches Veterinäruntersuchungsamt Detmold, Detmold, Germany*

Keywords: HN gene, L gene, paramyxovirus, PCR, U gene

Introduction and objectives
Paramyxoviruses (PMV) are an important cause of disease in snakes and have also been described in lizards and chelonians. They are most commonly associated with respiratory disease in infected animals. Characterization of reptilian PMV to date indicates that these viruses should be classified in the subfamily *Paramyxovirinae* in a new genus with the proposed name "Ferlavirus" (Kurath et al., 2004). However, the number of reptilian PMV characterized remains small, and little is known about the relationships between PMV from snakes, lizards, and chelonians. This study compared PMV from snakes, lizards and tortoises based on partial sequences of the L, HN and U genes, providing new information on the taxonomical position of these viruses. RT-PCRs targeting these genes were used for PMV detection from diagnostic samples and diagnostic methods were compared.

Materials and methods
PMV isolates from seven snakes, three lizards and one tortoise were included in this study. Additional diagnostic samples from over 300 snakes and a tortoise (*Geochelone pardalis*) were tested. RNA was prepared from infected cell cultures or samples. RT-PCRs targeting portions of the L and HN genes were carried out as described previously (Ahne et al., 1999). RT-PCRs targeting the U gene, which is believed to be unique to the ophidian PMV, were carried out as described (Kurath et al., 2004). Additional primers were designed to detect portions of the target genes from additional isolates, based on information obtained during the course of this study. All PCR products were sequenced and sequences analyzed and compared.

Results
Partial L gene sequences were obtained from all of the PMV isolates tested. Partial HN and full U gene sequences were obtained from all isolates except the tortoise isolate. Analysis of the sequences showed that the viruses were most closely related to one another and to ophidian PMV described previously, building a monophyletic group consistent with a separate genus within the subfamily *Paramyxovirinae* (Fig. 1). The viruses clustered into two groups according to sequences from all three genes. The PMV isolate from the tortoise clustered separately from the snake and lizard isolates, but within the proposed new genus. A comparison of different RT-PCRs for the detection of PMV in clinical samples from snakes showed that the nested RT-PCR targeting the L gene described previously (Ahne et al., 1999) is the most broadly reactive of the tested RT-PCRs and can be used for diagnostic purposes. This method is also much more sensitive than classical virus isolation techniques. Analysis of clinical samples showed that all clustered together within the proposed "Ferlavirus" genus. In several cases, snakes and a single tortoise (*G. pardalis*) were found to be concurrently infected with several different PMV.

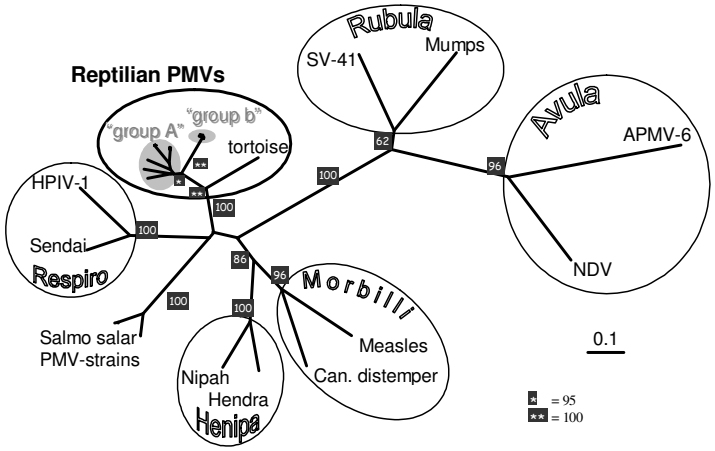

Figure 1. Phylogenetic tree of Paramyxovirinae based on partial L gene sequences

Discussion and conclusions
PMV from reptiles have previously been termed ophidian PMV since they are most commonly found in snakes and reptilian PMV characterized to date have almost only been isolated from snakes. The results of this study show that PMV from snakes and lizards are very closely related. Analysis of portions of 3 different genes show no indication of species specificity among these viruses. This is somewhat different in the case of the chelonian PMV examined here. The PMV isolate from a tortoise included in the study differed clearly from the squamate PMV, although it did appear to belong in the same genus. It was only possible to obtain sequence data from a portion of the L gene of this isolate, despite the use of a number of consensus primers, indicating that the HN and U genes differ more strongly from those of other reptilian PMV. Additional study is necessary to understand the relationship between this virus and the other reptilian PMV. Interestingly, we were also able to detect PMV in clinical samples from a *G. pardalis*. The partial L gene sequences obtained from this animal were most closely related to PMV from snakes, indicating that chelonians can be infected with the same PMV as other reptiles. In that case, however, three different PMV were detected, indicating repeat infection and a possible weakened immune system in that animal. The RT-PCR targeting the L gene is a useful tool for the diagnosis of PMV infection in reptiles. It is, however, a nested PCR, making it prone to contamination and time consuming. It also frequently results in multiple bands, making additional analysis of PCR products more difficult but also more important. Further improvement of molecular diagnostic methods for the detection of these important viruses in reptiles would be helpful.

Acknowledgements
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NEW SQUAMATID ADENOVIRUSES: PARTIAL PCR-SEQUENCE CHARACTERISATION OF THE FIRST LIZARD ISOLATES TOGETHER WITH OTHER SNAKE AND LIZARD FIELD SAMPLES

Tibor PAPP[1], Beth FLEDELIUS[2], Volker SCHMIDT[3], Győző L. KAJÁN[4], Inna ROMANOVA[1] and Rachel E. MARSCHANG[1]

[1]Institut für Umwelt- und Tierhygiene, Hohenheim University, Garbenstr. 30, 70599 Stuttgart, Germany;
[2] Small Animal Clinic „Hjortekaer", 2800 Kgs Lyngby, Denmark;
[3] Bird and Reptile Klinik of Leipzig University, 04103 Leipzig, Germany;
[4]Veterinary Medical Research Institute of the Hungarian Academy of Sciences, Hungária krt. 21., 1143 Budapest, Hungary

Keywords: adenovirus, coevolution, PCR, reptile, virus isolation

Introduction and objectives

Adenoviruses (AdVs) occur worldwide and have been described from a wide range of vertebrates. The family *Adenoviridae* currently has four accepted genera (*Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus*) representing the presumed mammalian, avian, reptilian and amphibian coevolved lineages respectively, and a fifth proposed genus (Ichtadenovirus) containing the only known fish AdV so far (1). Adeno(-like)viruses have been detected by conventional and molecular virological methods in various reptilian hosts, including squamatids (snakes and lizards), archosaurids (crocodiles) and chelonids (tortoises and turtles), mostly from captive bred animals. Antibodies have also been detected in wild snake populations, suggesting widespread distribution in nature (3).The clinical symptoms of AdV infection vary from enterohepatic inflammation to splenitis, nephritis, pneumonia or encephalopathy. However AdV infections in reptiles do not always result in a disease but are often inapparent.

To date the most useful tool for the quick diagnosis and characterisation of AdV infections (not only in reptiles) is PCR detection followed by direct sequencing as described by Wellehan et al. (5). We used this consensus nested PCR to detect AdVs from clinical samples of reptiles over a 3 year period of time and characterized a total of 17 squamatid AdVs, all clustering in (or near) the *Atadenovirus* genus. In most cases virus isolation was also attempted, mostly without success. In one group of helodermatid lizards we were however able to isolate two similar viruses. These helodermatid AdVs are the first lizard AdV isolates (4). Further genetic characterization of these isolates is currently underway.

Materials and methods

Diagnostic samples of oral/cloacal swabs from live animals and different tissues from dead animals from over 150 snakes and lizards and several tortoises have been tested for the presence of AdV by consensus nested PCR targeting the DNA polymerase gene (5) in our lab since 2006. PCR amplicons were directly sequenced and the sequences obtained were used for phylogenetic analysis. Positive samples were later tested with other PCRs targeting other portions of the virus genome and amplicons were sequenced. For over 80% of all diagnostic samples, virus isolation was also attempted on host-specific cell lines (TH-1, VH-2 or IgH-2). Cell culture dishes were passaged consequently 3 times at 2 week intervals. Those showing adenovirus-like CPE were tested with PCR and EM for the presence of AdVs. Verified isolates were further propagated on the homologous cell lines, virus titer was determined and virus particles were concentrated by ultracentrifugation.

Results

We have found AdVs in a total of approximately 40 diagnostic samples during the 3 year period. Lizard AdVs were detected from an emerald monitor (*Varanus prasinus*), 8 bearded dragons (*Pogona vitticeps*), 3 mexican beaded lizards (*Heloderma horridum*) and 15 Gila monsters (*Heloderma suspectum*). These latter 18 cases originated from a Danish zoo, where the helodermatid lizards were kept together with other reptiles. A number of the helodermatid lizards died in a sudden outbreak. A follow-up study was conducted during which several of the companion lizards and two leopard tortoises from the same enclosure gave negative PCR results months later, while some lizards were still positive. AdVs were detectable in freshly introduced Gila monsters for up to 1⅓ year after the outbreak. Interestingly, the sequences obtained from the viruses in Mexican beaded lizards differed from those of the Gila monsters (9% nt, 3% aa). These latter sequences were however identical to the Helodermatid AdV described in USA from the same host (Wellehan et al., 2004), concerning the partial polymerase gene region (272bp). AdVs were successfully isolated from two Gila monster samples and from a Mexican beaded lizard sample and propagated on iguana heart cell lines (IgH-2) with up to a titer of 10^7 $TCID_{50}$/ml. A short part (250 bp) of the hexon genes of the isolates were sequenced and show similar sequence divergence values (9% nt, 5% aa) to those found in the DNA polymerase gene. AdVs were also detected in several snake collections with no specific signs indicative of AdV infection. In most cases paramyxoviruses (PMVs) were suspected as the cause of disease, but PMV tests were negative. Two tiger pythons (*Python molurus*), a royal python (*P. regius*) and a common boa (*Boa constrictor*) had adenoviruses which were shown to be closely related to the previously fully characterised corn snake isolate (SnAdV-1) the "prototype" of reptilian AdVs (2), but were not identical to it. The greatest sequence divergence within this group was 12% and 8.5% for the partial polymerase gene nucleotide and amino acid sequences respectively. A distinctly different AdV (37% nt, 35% aa compared to SnAdV-1 partial polymerase) was found in an asp viper (*Vipera aspis*) that died suddenly. All new AdVs will be presented on a phylogenetic tree.

Discussion and conclusions

We have successfully isolated AdVs from lizards. The two highly similar helodermatid AdVs support the coevolution theory, and presumably represent one viral species. Now that these viruses have been isolated in cell culture, a larger amount of virus is available for serological studies and for further genomic characterisation. The low divergence among agamid AdVs suggest that the same type of AdV is widely distributed among the breeds in Europe and USA. The varanid AdV polymerase sequence differed the most from every other squamatid AdVs.

Concerning the snake AdV sequences, they both support and contradict the coevolution theory as the same sequences were detectable from different host species, whereas the phylogenetic grouping of some snake AdVs resembled the phylogenetic relationship of the host snakes.

The consensus nested PCR targeting the polymerase gene of AdVs is an excellent diagnostic tool and the most sensitive at the moment. However, the amplicons must be sequenced before interpreting the results, because false positives often occur with chelonian samples and because prey specific AdVs can be also amplified from carnivorous squamates.

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THE PHYLOGENETIC ANALYSIS OF AVIPOXVIRUSES IN NEW ZEALAND BIRDS

Hye-jeong HA, Laryssa HOWE, Maurice ALLEY, Brett GARTRELL

Institute of Veterinary, Animal and Biomedical Sciences (IVABS), Massey University, Palmerston North, New Zealand

Keywords: avipoxvirus, New Zealand birds, phylogenetic analysis

Introduction and objectives

Avipoxvirus is a member of Chordopoxvirinae which is a subfamily of virus family Poxviridae. It has been identified in more than 278 bird species in 20 orders (4). Since the first publication reporting avipoxvirus infection in 1844, it has been reported throughout the world both in captive and free-ranging birds (1). While avipoxvirus infection has caused economic loss in the poultry industry, recent attention has been on the impact on the biodiversity decline in free-ranging birds (3). Avipoxvirus is known to be endemic in New Zealand bird species (1, 2). It is an ongoing cause of mortality in endangered black robin (*Petroica traversi*) and shore plover (*Thinornis novaeseelandiae*) population (2). It is currently uncertain if these avipoxvirus infections represent native strains affecting depleted populations, or were introduced with their European avian hosts and have played a role in the decline of avian biodiversity. To our knowledge, no research has been conducted concerning the phylogenetic relationships between avipoxvirus strains in New Zealand. This study was designed to identify the phylogenetic relationships of avipoxvirus strains affecting the introduced and native bird species in New Zealand.

Materials and methods

Poxvirus-suspicious proliferative lesions in various New Zealand birds submitted for post mortem examination to IVABS, Massey Univeristy between 1992 and 2008 were collected. DNeasy Blood & Tissue kit (QIAGEN) was used to extract the DNA from the parafin-embedded tissues. The extracted DNA was screened by Polymerase Chain Reaction (PCR) assay for avipoxvirus 4b core gene using P1 and P2 primers (3). Among 24 samples that tested positive in the PCR assay, 15 cases were selected for the further gene sequencing by BigDye TM Terminator version 3.1 Ready Reaction Cycle Sequencing. The bird species included one North Island robin (*petroica australis*), two oyster catchers (*Haematopus unicolor*), one paradise duck (*Tadorna variegata)*, two South Island saddlebacks (*Philesturnus carunculatus carunculatus)*, one North Island saddleback (*Philesturnus carunculatus rufusater)*, four New Zealand shore plovers, one silvereye (*Zosterops lateralis*), one song thrush (*Turdus philomelos)*, one house sparrow (*Passer domesticus)* and one kereru (*Hemiphaga novaeseelandiae*). The sequences from 15 cases were combined with known avipox sequences published in GenBank for sequence alignment. The GenBank assession included in this study were vaccinia (M11079), fowlpox ATCC vaccine strain (AY453172), turkey field isolate/EU (AY530304), falcon field isolate/EU (AY530306), pigeonpox virus strains/EU (AY530303 & AY453177), agapornis field isolate/EU (AY530311), house finch/USA (DQ131896), robin/USA (DQ131902), canary field isolate/EU (AY530309), sparrow field isolate/EU (AY530308), and great tit pox isolate/EU (AY453174). Geneious Pro 4.5.4 was used for the nucleotide alignment and the phylogenetic tree was generated using bootstrap method.

Results

The size of resultant amplicons in PCR assay was approximately 540 base pairs (bp). The results of bootstrap analysis presented five distinctive clades. Agapornis isolate (AY530311) comprised clade 1 and one strain of pigeonpox (AY453177), house finch (DQ131895), and robin (DQ131902) comprised clade 2. Two shore plover isolates (46259 & 46260) originating from Mana Island and one South Island saddleback isolate belonged to a further clade (clade 3). Eleven of the 15 New Zealand avipoxvirus isolates belonged to clade 4, sharing same clade as fowlpox ATCC vaccine strain (AY453172) and turkey field isolate (AY530304). The kereru isolate, one strain of pigeonpox virus (AY530303) and falcon field strain (AY530306) comprised a separate clade (clade 5).

Discussion and conclusions

The results of this study demonstate that the majority of New Zealand avipoxvirus isolates belong to the same clade. The origin of these virus isolates remains uncertain as they share a great similarity with the fowlpox vaccine strain. There is also evidence that some avipoxvirus strains in New Zealand may have been introduced with the introduction of European bird species due to the genetic similarity with known European stains and the lack of similarity with American avipoxvirus strains. Although the impact of avipoxvirus infection in New Zealand bird populations has not been previously studied, it is apparent that many of New Zealand native bird speices are susceptible to the infection and there are more than two distinctive avipoxvirus clades. In the conservation management of native bird speices, surveillance for avipoxvirus infection and the identification of the virus strain should be taken into account.

Acknowledgement

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ASSOCIATION OF AVIAN BORNAVIRUS WITH TISSUE LESIONS IN PSITTACINES WITH PROVENTRICULAR DILATATION SYNDROME

Herbert WEISSENBÖCK[1], Karin SEKULIN[1], Tamás BAKONYI[2], Felix EHRENSPERGER[3], Norbert NOWOTNY[1]

[1]Department of Pathobiology, University of Veterinary Medicine Vienna, Austria;
[2]Faculty of Veterinary Science, Szent István University, Budapest, Hungary;
[3]Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zürich, Switzerland

Keywords: avian bornavirus; immunohistochemistry; *in situ* hybridization, RT-PCR, pylogenetic analysis

Introduction and objectives
Recently, two research groups provided independent evidence that proventricular dilatation disease (PDD), a well known, usually lethal condition of psittacine birds with world-wide occurrence is associated with presence of several genotypes of a novel virus species, provisionally termed Avian Bornavirus (ABV) (1, 2). This finding is intriguing, as it (1) suggests the likely etiology of this disease, which has been elusive for more than 30 years and (2) adds a novel species to the virus family Bornaviridae, which for a long time comprised a single virus species, Borna disease virus, the causative agent of a meningoencephalitis in sheep and horses in Central Europe.

Materials and methods
In this retrospective study, paraffin-embedded tissue samples from 20 psittacine birds of different species with clinically and histologically diagnosed PDD were examined by immunohistochemistry (with a crossreactive polyclonal antibody to BDV p 23 protein) and in situ hybridization (ISH) (with riboprobes targeting both, genomic RNA and mRNA of a part of the nucleoprotein gene of ABV genotype 2 and ABV genotype 4, respectively). Determination of the viral species present was achieved by RT-PCR and sequencing of the amplification products. In addition, 6 negative controls were also investigated. Generally, brain samples and samples of the upper digestive tract (crop, proventriculus, gizzard) were consistently examined. In some cases with robust viral signals in the CNS also other available tissues were included.

Results
Histological changes were present in the vegetative nervous system of the upper alimentary tract and in the brain. In crop, proventriculus and gizzard a mild to severe nonsuppurative ganglioneuritis was present. The brains showed nonsuppurative encephalitides of varying intensities. All these cases exhibited viral antigen in at least one of the investigated tissue locations. Usually, large to moderate amounts of viral antigen were present in the brain and intramural ganglia or vegetative nerve fibers of the digestive tract. Positive immunostaining was present in nuclei, perikarya and processes of neurons, but also in nuclei of glial cells. The distribution of viral signals in the brain was rather diffuse and random and varied from case to case. Occasionally, also non-neural cells, such as smooth muscle fibers or cells of the cardiac conduction system stained positive. Partial sequence analysis of the nucleoprotein- and matrix protein genes revealed either ABV genotype 2 or ABV genotype 4 infections in the majority of the cases. In two cases a previously not described nucleotide sequence was detected, leading to the assignment of a novel group of ABVs, ABV genotype 6 (3).

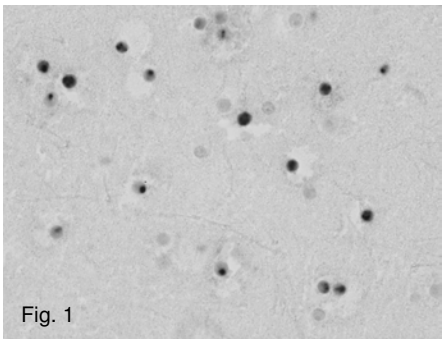
Fig. 1

Fig. 1. ISH showing distinct nuclear signals in cerebral neurons of a bird with PDD; ABV-4 probe complementary to genomic RNA.

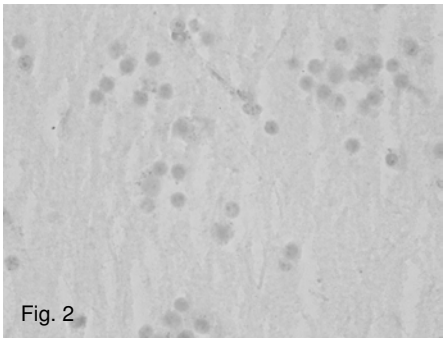
Fig. 2

Fig. 2. PDD-negative bird; no reaction with the ABV-4 probe.

Using ISH, the majority, but not all of the IHC- and RT-PCR-positive cases revealed distinct nuclear signals. In some cases viral signals of only one of the ABV genotypes investigated was present, in other cases signals of both genotypes were found. Generally, the signal of viral genome was more abundant than the signal of mRNA. Negative controls were negative with all assays used.

Discussion and conclusions
In this presentation we provide evidence that ABV viral proteins and nucleic acids are generally present in nervous tissues and less frequently also in non neural tissues of affected birds. These findings are in line with recent work from Germany (4) and add further support to the assumption that different genotypes of ABV are the etiologic agent of PDD, which obviously caused a persistent infection of the nervous system of the affected birds and initiated the resulting immunopathological changes.

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IDENTIFICATION OF A NOVEL HERPESVIRUS IN BAT USING IMPROVED RDV METHOD

Shumpei WATANABE[1], Ken MAEDA[2], Kazuo SUZUKI[3], Kentaro KATO[1], Yukinobu TOHYA[1], Tetsuya MIZUTANI[4], Hiroomi AKASHI[1]

[1] University of Tokyo, Graduate School of Agricultural and Life Sciences, Department of Veterinary Microbiology, Tokyo, Japan;
[2] Yamaguchi University, Faculty of Agriculture, Laboratory of Veterinary Microbiology, Yamaguchi, Japan;
[3] Hikiiwa Park Center, Wakayama, Japan;
[4] National Institute of Infectious Diseases, Department of Virology 1, Tokyo, Japan.

Keywords: bat, direct sequencing, herpesvirus, sequence-independent amplification, virus discovery

Introduction and objectives
Emerging infectious diseases pose a significant risk to public health. Methods for rapid detection of pathogens are needed to effectively treat these diseases. Recently, we developed new methods for the rapid determination of viral RNA sequences, RDV ver1.0, 2.0 and 3.0. We demonstrated that these methods were able to simultaneously detect cDNA fragments of many different viruses without using sequence specific primers (1-4). The RDV methods were further modified to reduce the candidate PCR primer sets, and it was applied to identify a virus isolated from a bat collected in Japan.

Materials and Methods
An insectivorous bat, *Miniopterus fuliginosus*, was caught in Japan. Its spleen was used to establish primary cell cultures. On the passage of the primary adherent cells, a cytopathic effect appeared. RNA was extracted from the supernatant of the cells, and subjected to the improved RDV method, RDV ver3.1 to identify the isolated virus. Design and scheme of RDV ver3.1 was summarized in Fig. 1.

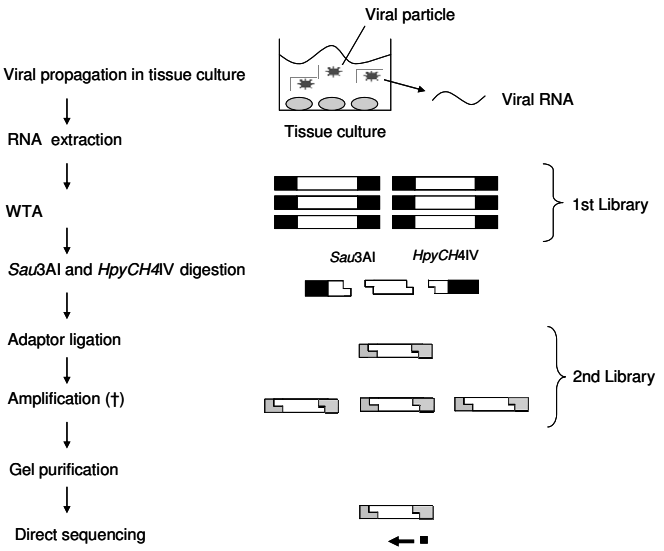

Fig. 1. Overall scheme for RDV ver3.1. WTA, whole transcriptome amplification. (†) with 64 RDV primer sets, in which the restriction enzyme (Sau3AI or HpyCH4IV) digested sequence and a few variable nucleotides were added to the 3' end of the adaptor sequence.

Results
Using the RDV method, several cDNA fragments were obtained, and these were homologous to the genes of Tupaia herpesvirus. To further confirm that the virus belongs to the Genus *Betaherpesvirus*, we designed new consensus-degenerate hybrid oligonucleotide primers targeting the betaherpesvirus gB gene, and PCR was performed. A fragment of about 1 kbp was amplified and subjected to sequencing analysis. BLAST search showed that the virus is a novel betaherpesvirus.

Discussion and conclusions
We isolated a novel virus from an insectivorous bat, and succeeded to identify the virus using the improved RDV method. This method has a potential to detect viral cDNA fragments of both known and unknown viruses rapidly.

Acknowledgements
We thank Dr. Shigeru Morikawa, of the National Institute of Infectious Diseases of Japan, for useful suggestions. We thank Ms. Momoko Ogata and Ms. Kyoko Kano for their assistance. This study was supported in part by grants from the Japan Society for the Promotion of Science, the Ministry of Health, Labor, and Welfare, and Ministry of Education, Culture, Sports, Science and Technology, Japan.

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DETECTION, DIVERSITY AND EPIDEMIOLOGY OF MARINE MAMMAL ASTROVIRUSES

James F. X. WELLEHAN[1], Rebecca RIVERA[2], Stephanie VENN-WATSON[3], Cynthia SMITH[3], Eric JENSEN[3], Frances M.D. GULLAND[4], Hendrik NOLLENS[1,2]

[1]Marine Mammal Health Program, College of Veterinary Medicine, University of Florida, P.O. Box 100126, Gainesville, FL 32610, USA;
[2]Hubbs-SeaWorld Research Institute, 2595 Ingraham St, San Diego, CA 92109, USA;
[3]U.S. Navy Marine Mammal Program Foundation, San Diego, CA 92106, USA;
[4]The Marine Mammal Center, Fort Cronkhite, CA 94965 USA

Keywords: astrovirus, consensus PCR, epidemiology, marine mammals, recombination

Abstract

Astroviruses are enteric RNA viruses belonging to the family *Astroviridae*. Electron microscopic analysis of fecal samples from one bottlenose dolphin (*Tursiops truncatus*), three California sea lions (*Zalophus californianus*), and one Steller sea lion (*Eumetopias jubatus*) showed astrovirus-like particles. Degenerate PCR primers were designed targeting conserved regions of astroviral genomes. Phylogenetic analysis of the resulting sequences revealed five distinct astrovirus species, significantly expanding known astroviral diversity. Recombination analysis showed a relatively recent recombination event may have occurred between a human and a California sea lion astrovirus, suggesting that both lineages may have been capable of infecting the same host at one point.

A serosurvey of dolphins using an ELISA demonstrated that dolphins greater than 25 years old had higher antibody levels than dolphins younger than 5, and samples collected in spring had higher antibody levels compared to the other three quarters ($P = 0.01$). Dolphin astrovirus (TtAstV-1) appears to be common, with epidemiology similar to astroviruses in other species. Serologic evidence of exposure was found in both managed-collection and free-ranging dolphins.

In humans, astroviruses are a leading cause of viral diarrhea in young children (Dennehy, 2001), and astroviruses have been found in 10% of diarrheic cats (Marshall et al, 1987). Although we were unable to attribute clinical signs to the presence of the astroviruses, it is likely that they have the potential to cause enteritis in marine mammals. Their stability in seawater and the diversity found in marine mammals suggests that the marine environment may be important in astroviral ecology.

Acknowledgements

This work was funded by research grant No. N00014-06-1-0250 from the Office of Naval Research to H.N. All sample collection protocols were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC# C233). We would like to thank Dr. Pam Yochem (Hubbs-SeaWorld Research Institute) and Dr. Judy St. Leger (SeaWorld San Diego); the staff of the U.S. Navy Marine Mammal Program for their assistance with sample collection.

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NOVEL MARINE MAMMAL VIRUSES: IMPLICATIONS FOR HUMAN AND ANIMAL VIRUS ECOLOGY

Hendrik H. NOLLENS[1], Rebecca RIVERA[2], Stephanie VENN-WATSON[3], James F.X. WELLEHAN[1]

[1]Marine Mammal Health Program, College of Veterinary Medicine, University of Florida, P.O. Box 100126, Gainesville, FL 32610, USA;
[2]Hubbs-SeaWorld Research Institute, 2595 Ingraham St, San Diego, CA 92109, USA.
[3]U.S. Navy Marine Mammal Program Foundation, San Diego, CA 92106, USA

Keywords: emerging, marine, novel, virus discovery, virus ecology

Virus discovery work in our laboratory has demonstrated an interconnectivity between marine and terrestrial viruses. Marine mammals have shown to be susceptible to disease syndromes similar to their terrestrial counterparts. We have identified novel astroviruses, coronaviruses, polyomaviruses, parainfluenzaviruses, and reoviruses in marine mammals that expand our knowledge of virus ecology and evolution, and have also identified terrestrial enteroviruses in marine mammals. Recombination between human and marine mammal astroviruses has been demonstrated. We are applying comparative genomics using these marine viruses to provide insights into pathogenicity factors of virus groups that are known to infect humans. Most emerging diseases are zoonotic in origin, and viruses in the marine environment are poorly understood. Our findings suggest that the marine ecosystem is an important component of terrestrial viral ecology, and understanding marine viruses may be of significance to human and terrestrial animal health.

Acknowledgements
This work was funded by research grant No. N00014-06-1-0250 from the Office of Naval Research to H.N. All sample collection protocols were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC# C233). We would like to thank Dr. Frances Gulland (The Marine Mammal Center), Dr. Pam Yochem (Hubbs-SeaWorld Research Institute) and Dr. Judy St. Leger (SeaWorld San Diego); the staff of the U.S. Navy Marine Mammal Program for their assistance with sample collection.

MOLECULAR CHARACTERIZATION OF A NOVEL CORONAVIRUS, INDUCING FELINE INFECTIOUS PERITONITIS (FIP)-LIKE LESIONS IN FERRETS

Annabel G. WISE, Matti KIUPEL, April K. CLARK, Roger K. MAES

College of Veterinary Medicine, Michigan State University, E.Lansing, MI 48824 USA

Keywords: enteric, ferret coronavirus, systemic, virology

Introduction and objectives

A systemic disease, characterized by FIP-like clinical signs and lesions, has recently been observed in ferrets across the U.S. and Europe (2). Clinical findings include anorexia, weight loss, diarrhea and the presence of large palpable intra-abdominal masses. Similar to FIP in cats, the lesions consist of widespread nodular foci on serosal surfaces and within the parenchyma of the abdominal and thoracic organs. A coronavirus etiology, suspected based upon immunohistochemical (IHC) staining with feline coronavirus antibody, was confirmed by our sequencing data of a portion of the spike gene (2). The virus was tentatively designated ferret systemic coronavirus (FSCV). The objective of this study was to more definitively characterize FSCV at the molecular level and to elucidate its genetic relationship to ferret enteric coronavirus (FECV), a novel group 1 coronavirus associated with epizootic catarrhal enteritis in ferrets (5). We report here the determination and comparative analysis of the approximately 8.6 kb long 3' genomic end sequences of these two ferret coronaviruses.

Materials and methods

Tissue samples were obtained from a ferret that had exhibited clinical signs and lesions indicative of FSCV infection and was positive by IHC for coronavirus antigen. Total RNA was extracted from tissues of this ferret and also from a fecal sample from a ferret diagnosed with FECV infection. The systemic and enteric ferret coronavirus strains analyzed were designated as FSCV MSU-1 and FECV MSU-2, respectively.

Partial sequences of the S and M-N gene regions were obtained using consensus coronavirus RT-PCR primers. To bridge the gap between the 3' end of the S gene and the 3' end of the M gene, primers were designed from obtained partial sequences of the S and M genes for an S-M RT-PCR spanning an approximately 2 kb region. The remaining 3' end sequence of the genome was obtained by performing a 3' RACE. To derive the remaining S gene sequence (~3.2 kb), upstream of the already sequenced 3' end of the gene, 3 overlapping PCR fragments were amplified sequentially by designing degenerate forward primers via the CODEHOP (consensus-degenerate hybrid oligonucleotide primer) strategy and specific reverse primers from already determined sequence data. The PCR products were purified and sequenced bidirectionally. Partial S gene and ORF 3 region sequences of 2 additional FSCV and 1 FECV cases were also obtained.

Sequence assembly and analyses, including open reading frame (ORF) searches and multiple alignments of nucleotide and predicted amino acid sequences, were done with the Lasergene package. Phylogenetic trees were constructed with the Treecon software package. Sequence alignment data was converted to the Treecon format using the ForCon software.

Results

Sequences of the distal third of the genomes of FSCV MSU-1 and FECV MSU-2 were derived. Each sequence comprises the 3' terminus of the pol1b gene, the full coding sequences of structural protein genes S, E, M and N, non-structural protein (nsp) genes (ORF 3, 3x-like, and 7b), and partial sequence of the 3' untranslated region (UTR).

The genomic organization observed for each virus was consistent with that of a typical coronavirus. Pairwise alignment of the sequences showed an overall similarity of 87.1%. For individual ORFs, the pol 1b, M, N, 3x and 7b genes exhibited the highest nucleotide sequence similarities (96.1-97.5%). The ORF 3 region contained a single 3c-like gene for both viruses. The translation product of this gene is truncated in FSCV MSU-1. Significant divergence between the two viruses was observed in the S and the E genes, with nucleotide sequence similarities of only 79.5% and 88%, respectively. Amino acid substitutions between FSCV-MSU-1 and FECV MSU-2 appeared randomly distributed within the S protein sequence. Analysis of partial S sequences of additional systemic and enteric ferret coronaviruses showed that 21 amino acid substitutions between FSCV and FECV strains were conserved within a virus pathotype.

Discussion and conclusions

Ferret systemic coronavirus (FSCV) infection has clinical and pathological features that are very reminiscent of those induced by FIP virus (FIPV) in cats. Partial genomic characterization of FSCV strains available to us indicated, however, that FSCV strains differ more significantly from ferret enteric coronavirus (FECV) strains than is the case between feline enteric coronavirus (FeCoV) and FIPV strains.

Candidate feline coronavirus virulence genes include the S gene and group-specific genes 3abc, 7a and 7b (3). The C-terminal 582-AA regions of the S genes of an FIPV and a related FeCoV strain showed only 10 amino acid differences (4). A "D-to-A" substitution, upstream of the putative fusion peptide, was considered to play a role in the highly enhanced macrophage tropism of FIPV strains (4). Interestingly, FSCV MSU-1 has an alanine residue in this position also, while FECV MSU-2 has a serine residue.

FIPV deletion mutants in ORFs 3abc or 7ab showed an attenuated phenotype in cats (3). In contrast, a systemic canine coronavirus (CCV) strain had a truncated 3b protein (1). Consistent differences between the ORF 3 region of FSCV and FECV strains have not been detected in the strains analyzed thus far. The 7b protein sequence of FECV MSU-2 is 100% identical to the sequence of previously reported enteric strain FECV-MSU1 (5). The 7b nsp of FSCV MSU-1 is only 93.6% similar to the proteins of the two enteric strains. The 7a gene is not found in both FSCV MSU-1 and FECV MSU-2. In its place instead is a 3x-like gene with sequence homology to the CCV Insavc-1 strain 3x pseudogene which is part of the ORF 3 gene cluster of this strain. This unexpected location of the 3x-like gene appears to be a consistent feature among ferret coronaviruses, regardless of pathotype. In summary, FECV and FSCV are group 1 coronaviruses that segregate into their own distinct cluster within the group, but appear to be two genetically distinct viruses.

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SHREWS CARRY A VARIETY OF NOVEL HANTAVIRUSES: EVIDENCE FOR UNUSUAL BIOLOGICAL CHARACTERISTICS, COMPARED TO RODENT-BORNE HANTAVIRUSES

Norbert NOWOTNY[1], Ralf DÜRRWALD[2], Jolanta KOLODZIEJEK[1], Tamás BAKONYI[3,1], Armin DEUTZ[4], Herbert WEISSENBÖCK[5], Hans HOMOLA[1], Helga LUSSY[1], Tibor CSÖRGŐ[6], Gábor RÁCZ[7], Emőke FERENCZI[8], Barbara HERZIG[9], Boris KLEMPA[10], Detlev H. KRÜGER[10]

[1]Zoonoses and Emerging Infections Group, Clinical Virology, Department of Pathobiology, University of Veterinary Medicine, Vienna, Austria;
[2]IDT Biologika GmbH (IDT), Dessau-Roßlau, Germany;
[3]Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Budapest, Hungary;
[4]Government Veterinary Surgeon, Regional Administrative Authority, Murau, Austria;
[5]Institute of Pathology and Forensic Veterinary Medicine, Department of Pathobiology, University of Veterinary Medicine, Vienna, Austria;
[6]Department of Anatomy and Cell Biology, Faculty of Science, Eötvös Loránd University, Budapest, Hungary;
[7]Department of Biology, University of New Mexico, Albuquerque, New Mexico, U.S.A.;
[8]National Center for Epidemiology, Budapest, Hungary;
[9]Natural History Museum Vienna, Vienna, Austria;
[10]Institute of Virology, Charité University Hospital, Berlin, Germany

Keywords: central Europe, novel hantaviruses, phylogeny, RT-PCR, sequencing, shrews

Introduction and objectives
Hantaviruses belong to the genus *Hantavirus* within the *Bunyaviridae* family. They establish persistent, non-pathogenic infections in rodents, which shed the virus in secreta and excreta, which are the sources of incidental human infections. In human beings, hantaviral infections may result clinically in hemorrhagic fever with renal syndrome and hantavirus cardiopulmonary syndrome, respectively. With very few exceptions, genetically distinct hantaviruses are usually carried by single rodent species.

The viral genome of hantaviruses consists of 3 unique molecules of negative sense ssRNA, designated L (large), M (medium) and S (small). They encode the viral RNA polymerase (L protein), envelope glycoproteins (Gn and Gc, or G1 and G2), and nucleocapsid protein (N) in the virus-complementary sense RNA.

Recently several novel hantaviruses have been identified in different species of insectivores worldwide. Their potential to cause disease in human beings is still unclear.

The aim of our study was to investigate the occurrence, distribution and genetic relatedness of hantaviruses in shrews in three central European countries (Austria, Germany and Hungary).

Materials and methods
Eighty shrews (52 from Germany, 20 from Hungary, and 8 from Austria) of five different species (*Sorex araneus*, *Sorex minutus*, *Crocidura leucodon*, *Crocidura russula*, and *Neomys anomalus*) were collected, visually typed, and dissected.

Lung and liver samples of each shrew were subjected to RT-PCR analysis employing oligonucleotide primers targeting a highly conserved region within the hantaviral L segment. RT-PCR amplification products were sequenced, and phylogenetic analysis was carried out with the obtained sequences; sequences of all other insectivore hantaviruses known today and deposited in GenBank database were also included in the phylogenetic tree as well as sequences from the main rodent-borne hantaviruses. The phylogentetic investigations were performed using the Phylogeny Inference Program package, PHYLIP.

Hantavirus-positive shrews were – in addition to visual typing – also typed by PCR and subsequent sequencing of the complete cytochrome b gene.

Results and discussion
Hantaviral sequences were obtained from 3 German, 6 Hungarian and 2 Austrian *Sorex araneus* shrews, as well as from one *Neomys anomalus* shrew originating from the city of Murau, Styria, Austria. Since this is the first description of a hantavirus from *Neomys anomalus* this virus has – according to the current hantavirus nomenclature – been provisionally designated Murau virus.

Despite the large number of *Crocidura* species investigated no hantaviral sequences were found in them.

Phylogenetic analysis revealed the existence of several distinct hantaviruses within the species *Sorex araneus* clustering according to their geographic origins. Interestingly, the partial L gene sequence of the *Neomys anomalus*-derived hantavirus proved to be closer related to the Austrian *Sorex araneus* hantavirus sequences than the *Sorex araneus* hantavirus sequences from the neighbouring European countries to each other. This observation could indicate different epidemiological characteristics of shrew-borne hantaviruses compared to rodent-borne hantaviruses, which generally cluster according to their rodent host species.

In my presentation the current hantavirus nomenclature will also be critically evaluated.

HIGH PREVALENCE OF HEPATITIS E VIRUS INFECTION IN PIGS IN DIFFERENT AREAS OF THE UK

Francesca MARTELLI[1], Sylvia S. GRIERSON[1], Alessandra BERTO[1], Nadia INGLESE[1], Catherine MCCREARY[2], Charlotte DAWSON[2], Amanda NEVEL[2], Malcolm BANKS[1]

[1]Veterinary Laboratories Agency Weybridge, United Kingdom;
[2]Royal Veterinary College, London, United Kingdom

Keywords: age classes, HEV, prevalence

Introduction and objectives
HEV (Hepatitis E Virus) is the sole member of the genus Hepevirus in the family Hepeviridae, and causes acute hepatitis in humans (Huang et al., 2002). The virus is transmitted via faecal contamination of drinking water in developing countries, whilst in developed countries the disease occurs sporadically, mainly affecting people returning from travel in endemic areas. Autochthonous cases have been reported with increased frequency in developed regions, suggesting the existence of a reservoir of the infection. Recently, it has been demonstrated that the virus can infect animals, and pigs are considered to be a reservoir of the infection (Meng et al., 1997). The infection is asymptomatic in pigs and is widely distributed in the swine population worldwide. The prevalence of HEV infection varies according to the age of the animals (Fernandez-Barredo et al., 2006). The aim of this study was to investigate the prevalence of HEV excretion in pigs in farms located in Yorkshire-Humberside and East Anglia in the UK and to genetically characterise the strains identified.

Materials and methods
Ten farms were visited in each area during 2007 (Yorkshire-Humberside) and 2008 (East Anglia). Five samples were collected from weaners, growers, fatteners, and adult sows on each farm; a total of 390 faecal samples were collected. RNA was extracted from the faecal samples and amplified with a RT-Nested-PCR protocol (Huang et al., 2002). The data were analysed with a chi-squared test, using SPSS software for Windows 15.0. HEV prevalence was analysed in relation to age class, area of sampling, housing system and herd system. Positive samples were sequenced and phylogenetic analysis was carried out with PHYLIP (version 3.67) using DNADIST and NEIGHBOR. The dendrogram was visualised in Treeview (version 1.6.6).

Results
The mean prevalence for Yorkshire-Humberside was 21.5%, and for East Anglia 10.4%, and the difference was statistically significant (p=0.003). All farms had at least one positive sample, with the exception of the two outdoor herds, where all samples were negative. The difference in the prevalence between outdoor and indoor herds was significant (p=0.028). As indicated above, the difference in the regional prevalence was significant but when the data from the two outdoor herds were removed, the difference was no longer significant (p>0.05). HEV positive samples were detected within all four age classes (Figure 2). In both studies the age class of growers showed a higher prevalence (44% and 22% respectively). The difference between the prevalence in the age classes was significant (p=0.000), both when area data was analysed independently and when age class data from the two studies were analysed collectively. When considering the herd system (batch or continuous, data available only for samples collected in 2008), no significant difference was detected between the two prevalence values (p=0.101). All strains sequenced belonged to genotype 3e, and are closely related to other swine and human strains circulating in the UK and Europe (Figure 1). Sequences from animals from within the same herd tended to cluster together, and some sequences from animals of the same age class within the same herd were shown to be 100% identical.

Discussion and conclusions
The prevalence of HEV reported here is consistent with those that have been found in other European surveys. There was no obvious difference in the density of herds between the two regions that would account for the higher prevalence that was detected within the Yorkshire-Humberside area. However, the inclusion of two outdoor herds within the second study would appear to have influenced the prevalence that was found within East Anglia. The apparent HEV negative status of the outdoor units is worthy of further investigation, but may have been affected by the greater exposure to UV compared to the indoor herd samples. Animals shedding the virus were found within all age classes. The growers have been confirmed to be the age class with the higher prevalence of infection, as has been extensively reported worldwide. Despite a lower prevalence within the fatteners, the finding that animals close to the food chain are shedding virus, raises concerns about public health. The detection of virus within sows has already been reported in other surveys, and could explain the persistence of HEV within herds. No data relating HEV prevalence to the herd system are available in the literature. No difference between batch and continuous systems was found in this study but further work will be required to confirm that this finding is not an effect of a small sample set. The close homology of strains circulating in the same herd and within animals of the same age class further support the persistent circulation of closely related strains within a herd. The homology between swine and human strains circulating in the UK provides further evidence to the hypothesis of zoonotic transmission of HEV.

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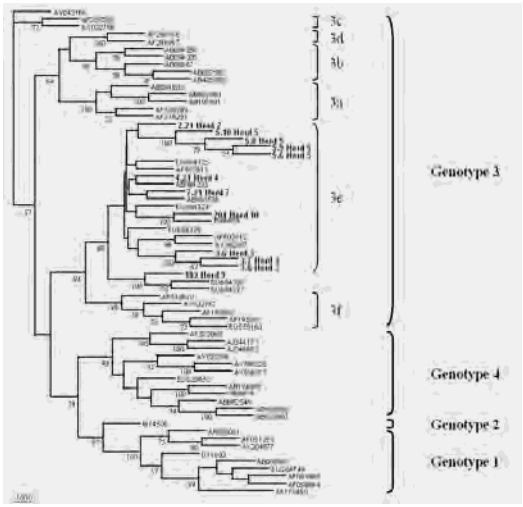

Figure 1: Phylogenetic tree of the strains identified.

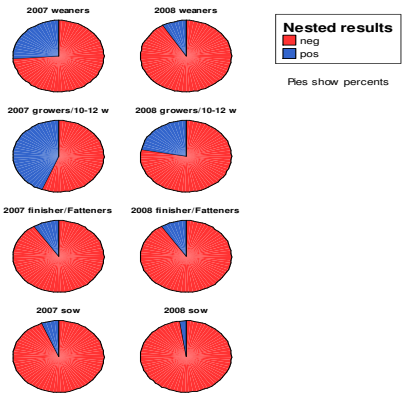

Figure 2: Age related prevalence of HEV by year.

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MOLECULAR EPIDEMIOLOGY OF HEPATITIS E VIRUS IN SWINE AND WILD BOAR IN GERMANY

Edmilson F. OLIVEIRA-FILHO, Barbara R. BANK-WOLF, Heinz-Jürgen THIEL, Matthias KÖNIG

Institut für Virologie, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, Giessen, Germany;

Keywords: HEV, phylogenetic analyses, RT-PCR, zoonosis

Introduction and objectives

Hepatitis E virus (HEV) is a non-enveloped positive strand RNA virus with a size of 27-32 nm. The genome of approximately 7.2 kb contains three open reading frames (ORF). HEV belongs to the genus *Hepevirus* within the family *Hepeviridae*. HEV can be further subdivided into four genotypes. It has been shown that the prevalence of certain genotypes differs according to geographical regions. The virus was first identified 1980 in India as source of a water borne outbreak in human population.

Hepatitis E is an emerging acute liver disease in humans distributed worldwide and the virus has also been reported in both domestic and wild animals (Meng, 2009). Previously the disease was considered endemic only in developing countries, but evidence accumulates that it is widespread in developed countries (Dalton et al., 2008). The prevalence rates differ considerably between countries and continents. Most infections with HEV take a subclinical or mild course. Infection in pregnant women is more severe with remarkably high morbidity and mortality rates (Dalton et al., 2008; Meng, 2009).

In Germany HEV has been reported in humans, including recent autochthonous cases (genotypes 3 and 4), and is now considered endemic (Wichmann et al., 2008). HEV has been detected in 5.3% of the wild boar population in northern Germany (Kaci et al., 2008). Natural infection in domestic pigs was first reported in 1997 in the United States (Meng, 2009) and in the following in many countries such as China, India and European countries. HEV was shown to be endemic in Spanish swine herds since 1985 (Casas et al., 2009). Until date HEV specific RNA has not been reported in domestic swine in Germany.

In the present study we report the presence of HEV specific RNA in domestic swine in Germany. Moreover we detected HEV in free-living wild boars from Hesse State.

Materials and methods

A total of 105 porcine fecal samples collected between 2003 and 2006, additionally 120 wild boar sera from 2009 were examined. RNA was isolated from both feces (swine) and sera (wild boar) using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. RT-PCR was performed as described (Lee et al., 2007). Positive samples were cloned using TOPO TA cloning kit (Invitrogen). Sequences were compared to Genbank entries and phylogenetic analyses performed using the HUSAR package (DKFZ, Heidelberg). Phylogenetic distances were calculated (Kimura-2-parameter method) and trees generated based on the neighbor-joining method. A bootstrap analysis with 1000 replicates was included. Branch lengths are proportional to genetic distances.

Results

A fragment of 266 bp from the capsid gene region of HEV was amplified in a single fecal sample from a domestic swine (0.95%) and from a number of sera from wild boar. Nucleotide sequence analyses were performed of 241 b from two samples demonstrating a difference of 17.28% between the viral sequences from domestic pig and wild boar at the nucleotide level. Phylogenetic analysis demonstrated that both sequences belong to genotype 3 (Fig. 1).

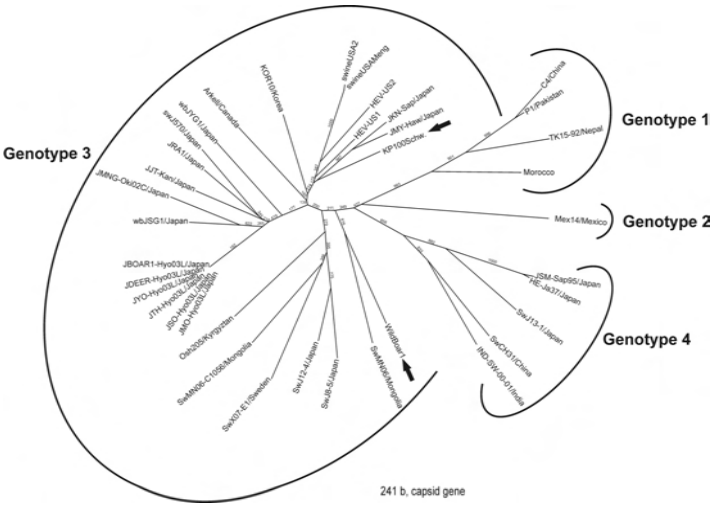

Fig. 1. Phylogenetic tree of HEV isolates based on 241 b of the capsid protein region. The sequences determined from two samples in this study are indicated with arrows.

Discussion and conclusions

Hepatitis E represents an underestimated zoonotic disease endemic in several industrialized countries. We report for the first time the detection of HEV specific RNA in domestic swine in Germany. The presence of HEV in wild boar has previously been reported in many countries including Germany (Kaci et al., 2006).

Prevalence data are preliminary so far with only a limited number of samples tested from different materials. However the virus appears to be more widespread in the wild boar population in comparison to domestic swine. It is unlike that this is due to different amounts of viral RNA in sera compared to fecal samples, since HEV can be detected more frequently and for longer periods in feces in comparison to blood samples. Additional studies should be conducted to address this point.

It has been suggested that domestic pigs as well as wild boar represent natural reservoirs for HEV (Meng et al., 2009). However the role of these animals in the epidemiology of the disease is not clear. Further studies are necessary to improve the understanding of virus transmission as well as to estimate the zoonotic risk.

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INFECTION OF DOMESTIC PIGS WITH A HUMAN *HEPATITIS E VIRUS* STRAIN: SYMPTOMS, PATHOHISTOLOGY AND DETECTION OF VIRAL RNA IN DIFFERENT ORGANS AND TISSUES

Christine BÄCHLEIN[1], Frauke SEEHUSEN[2], Sven PISCHKE[3], Heiner WEDEMEYER[3], Wolfgang BAUMGÄRTNER[2], Beatrice GRUMMER[1]

[1]Institute of Virology, University of Veterinary Medicine Hannover, Hannover, Germany;
[2]Department of Pathology, University of Veterinary Medicine Hannover, Hannover, Germany;
[3]Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany

Keywords: animal experiment, hepatitis E virus, pigs

Introduction and objectives
Hepatitis E virus (HEV) is the causative agent of a fecal-oral transmitted acute and self limiting hepatitis affecting mostly adults with low mortality. In Southeast Asia HEV is responsible for the majority of acute cases of hepatitis in humans, whereas in industrialized countries it is not considered as a major health problem (Purcell and Emerson, 2008). Nevertheless, antibodies directed against HEV can be found in humans all over the world and HEV isolates from pigs show high similarity to those recovered from humans (Meng et al., 1997). This led to the conclusion that domestic pigs and wild boar represent reservoir hosts for HEV worldwide.

The objective of this study was to analyze HEV infection and the impact on the health status of the reservoir host as well as the relevance of zoonotic transmission of HEV via pork meat.

Materials and methods
Five postweaning pigs were infected with an HEV isolate recovered from a male immunosuppressed patient being chronically infected with HEV. For this purpose, each animal received two milliliters of HEV positive human serum intravenously. Viremia in the patient was confirmed by reverse transcription (RT) PCR. Two mock-infected pigs served as a control. Clinical examination and determination of the body temperature was done once a day. Blood was taken twice a week and fecal samples were collected daily. In intervals of seven days post infection (p. i.) one animal was euthanized intravenously with pentobarbital. Five weeks p. i. the last HEV-infected animal as well as both control animals were euthanized, necropsied and examined for macroscopic and histologic lesions. Fresh organ samples including liver, bile, mesenteric lymph nodes and skeletal muscle were taken for RNA extraction and RT PCR.

Results
None of the pigs developed fever or showed any clinical signs during the experiment, except for one control animal which exhibited mild intermittent diarrhea. Macroscopically, no alterations were observed in the liver in any animal. Histopathological examination revealed a moderate lympho-histiocytic hepatitis and pericholecystitis in the pig euthanized six days p. i. and a mild lympho-histiocytic hepatitis in all the other animals, including the controls. Other histopathologic lesions found in lymph nodes, lung and kidney suggested an infection with *Porcine circovirus 2* (PCV2), a common and mostly subclinical infection in domestic pigs. This could be verified in all seven pigs by PCR, immunohistochemistry and *in situ*-hybridization. In order to find out whether HEV replicated successfully in the pigs, serum, feces and organs were submitted to phenol-chloroform extraction. RNA was reverse transcripted into cDNA and PCR was performed. The resulting product represented a 197 bp long amplicon located in the second open reading frame (ORF) which encodes for the capsid protein. Two animals exhibited viremia, in both cases viral RNA was demonstrated at one date of sampling. In another two out of five infected animals (necropsied on 6 and 27 dpi) HEV RNA could be detected in the liver, bile, mesenteric lymph node and *musculus psoas major* (filet muscle). Sequencing revealed HEV genotype 3 which is most common in western countries. Multiple alignment was performed and showed 90,8 % nucleotide identity between the virus isolated from the human patient and recovered from the infected pig 6 days p. i. and 96 % nucleotide identity between the human and the porcine virus from 27 days p. i. On amino acid level both porcine isolates exhibited 100 % identity with the original human strain.

Discussion and conclusions
The experiment shows that a human derived HEV strain is able to replicate in pigs which emphasizes the (antropo-) zoonotic character of this virus. Domestic pigs may serve perfectly as reservoir hosts as they do not exhibit any clinical symptoms underlining the good adaptation of the virus to its natural host. The short duration of viremia indicates that serum samples may not be the diagnostic material of choice to examine the viral status of domestic pigs. Although no macroscopic lesions could be observed, the histological findings concerning the liver suggest that HEV contributes to development of a mild hepatitis. The detection of HEV RNA in the filet muscle gives strong evidence that a viral transfer to humans is possible via the consumption of raw or undercooked meat. However, the impact of HEV on animal production and its possible role in human disease remain to be established.

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FIRST DISCOVERY OF HEV GENOTYPE 4 IN SWINE IN EUROPE THROUGH SCREENING OF FECAL SAMPLES, THE NETHERLANDS AND BELGIUM

Renate HAKZE-VAN DER HONING[1], Els VAN COILLIE[2], Wim H. M. VAN DER POEL[1]

[1]Central Veterinary Institute, Wageningen University and Research Centre, Lelystad, The Netherlands
[2]ILVO, Institute for Agricultural and Fisheries Research, Melle, Belgium

Keywords: hepatitis E virus, HEV, rRT-PCR

Introduction and objectives
Hepatitis E virus (HEV) is a small non-enveloped RNA virus of approximately 7.2 kb which has been classified within the family *Hepeviridae*, genus *Hepevirus*. Hepatitis E virus is an important cause of acute human hepatitis in regions with inadequate water supplies and poor sanitary conditions. Four different HEV genotypes (1-4) have been indentified. Zoonotic food-borne transmission of genotype 3 after eating meat from domestic swine, wild boar or deer has been reported (Tei et al., 2003). In developed countries HEV infection was regarded as travel-related only for a long time. However, an increasing number of indigenous HEV cases have recently been reported from developed countries. In contrast to what is seen in endemic areas in Asia and Africa, where the infecting viruses belong to genotypes 1 and 4, the indigenous infections in the US and Europe to date are caused by genotype 3 strains. HEV sequences in swine closely related to human sequences from the same region are reported for HEV3 and HEV4 in Europe and Japan respectively, indicating pigs are the likely reservoir of the indigenous infections in these regions. To study HEV prevalences and to characterize HEV strains in swine the Netherlands and Belgium, individual swine fecal samples were regularly collected from swine at slaughterhouses, and tested for HEV using molecular methods.

Materials and methods
In total 117 and 115 individual fecal samples were collected from slaughter house pigs in the Netherlands and Belgium respectively. All samples were tested using real time RT-PCR as described by Jothikumar et al., and all positives were sequenced on the ORF2 region of the genome. Obtained ORF2 sequences were analysed phylogenetically and compared to detected and published sequences of HEV genotypes from humans and swine.

Results
Hepatitis E virus prevalences in the individual swine fecal samples at slaughter were found to be 14% and 7% in the Netherlands and Belgium, respectively. Phylogenetic analyses revealed that all Dutch strains belonged to genotype 3. These strains could be divided in 3 different sub groups. Most of them belonged to subgroup 3e, two belonged to 3a and one belonged to 3f. Some sequences were closely related to sequences found in human patients. The HEV strains found in swine in Belgium belonged to genotype 3f and genotype 4c.

Discussion and conclusions
The detection of HEV genotype 4 is a striking observation. It is the first discovery of genotype 4 strains in swine in Europe. This seems an indication that genotype 4 in swine has spread to Europe. As a results, apart from zoonotic HEV genotype 3 infections, we can now expect zoonotic HEV genotype 4 infections in Europe also. It has been reported from Japan that HEV4 infections in humans run a more severe clinical course, demonstrated by significantly higher peak aminotransferase levels (Ohnishi et al., 2006). Therefore the emergence of HEV4 in swine in Europe may have an important public health impact.

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PHYOGENETIC ANALYSIS OF PARTIAL AND COMPLETE GENOME SEQUENCES OF HUNGARIAN HEPATITIS E VIRUS STRAINS WITH ANIMAL ORIGIN

Petra FORGÁCH[1], Gábor REUTER[2], Helga LUSSY[3], Norbert NOWOTNY[3], Tamás BAKONYI[1, 3]

[1]Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Budapest, Hungary;
[2]Regional Laboratory of Virology, Baranya County Institute of State Public Health Service, Pécs, Hungary;
[3]Zoonoses and Emerging Infections Group, Clinical Virology, Department of Pathobiology, University of Veterinary Medicine, Vienna, Austria

Keywords: hepatitis E virus (HEV), phylogenetic analysis, viral hepatitis, zoonoses

Introduction and objectives
Hepatitis E Virus (HEV) is an enterically transmitted human pathogen. Animal reservoirs are considered to play an important role in the maintenance of the virus and in the spread of HEV to humans. In previous studies, HEV RNA was detected by reverse transcription-polymerase chain reaction in samples of animal origin (wild boar, roe-deer, red-deer and domestic swine) collected in Hungary. Phylogenetic analysis of partial sequences of selected Hungarian strains and of a complete genome sequence reveals the genetic diversity of HEV and provides more information on the evolution and genetic relations of the potentially zoonotic virus.

Materials and methods
Between January 2004 and May 2009, 702 samples were collected in Hungary in order to investigate the presence of HEV in domestic and wild animals. Liver and faeces samples of domestic swine, wild boar, roe deer, red deer, cattle, sheep, goat, as well as rats, mice, hamster and common shrew were investigated. For the detailed phylogenetic analysis, 16 HEV positive samples were chosen, considering the host purposely to investigate at least one sample originated from each host species that was found positive.

In the reactions all together 17 primerpairs were applied producing overlapping sequences. Two sets of primerpairs (3), which were also used for diagnostic purposes, were used for the phylogenetic analysis of partial genome sequences in the case of 16 (ORF2 region) and 5 (ORF1 region) selected strains. The other 15 primerpairs were designed in order to determine the nearly complete genome sequence of a selected Hungarian strain.

Results
For detailed phylogenetic investigation 11 samples of domesticated swine, 2 samples of wild boar, 2 viruses of roe-deer and one sample collected in red deer were chosen. The investigated Hungarian strains belong to 3 different subgroups of the 3rd genogroup of HEV. The strains show high similarity of HEV strains detected in both human and animals from different countries. In a case of a selected strain a 7189 bp long sequence was determined. By the SimPlot analysis, two characteristic regions of the complete genome were identified.

By two analyzed regions of the genome sequence (ORF1 and ORF2), the investigated Hungarian HEV strains with animal origin belong to the *a*, *e* and *h* subgroups of the 3rd genogroup of HEV, showing similarity to HEV strains detected in both human and animal, collected in other countries. In the subgroups, the Hungarian viruses form distinct branches. Phylogenetic trees based on the deduced amino acid sequences are similar to the nucleotide sequence trees. The analysis of the deduced amino acid sequences also revealed, that the majority of the mutations are silent mutations.

The nearly complete genome sequence of a selected Hungarian HEV strain shows the highest similarity (98%) to a Japanese HEV strain of human origin. The Hungarian strain also closely related to other European viruses: the similarity is 84-85% with Spanish strains of swine origin, 85% with a Swedish strain of swine and 83% with a French HEV strain of human origin (1, 2, 4).

By the detailed investigation of the complete genome of HEV, all 3 ORFs were analyzed. At nucleotide level, the Hungarian strain shows 78-87% (ORF1), 83-91% (ORF2) and 91-98% (ORF3) similarity to the strains of the 3rd genogroup. At amino acid level, the similarity of the Hungarian strain to the 3rd genogroup strains is 90-94% (ORF1), 96-98 % (ORF2) and 90-97% (ORF3).

By the SimPlot analysis of the nearly complete genome sequence, we determined two characteristic regions of the HEV genome. The most variable region is the poly-proline hinge (PPH) coding site of ORF1, the most conserved is the capsid protein coding site of ORF2.

Discussions and conclusions
The Hungarian HEV variants belong to 3 subgroups of genotype 3 showing high diversity in their genome sequence. However all 3 ORFs were found suitable for phylogenetical analysis, the PPH coding region (ORF1) seems to determine mostly the genetic relations between the HEV strains. The reason of the variability is the difference in the length of the PPH site. Comparing the 3rd genogroup sequences to the sequences of the more ancient 1st and 2nd genogroups, the difference in the length of the PPH region is occurred by the insertion of numerous nucleotides.

The most conserved region was found within the ORF2, at the putative capsid protein coding site. Despite the high similarity (92-98%) between the investigated sequences at the most conserved region of the genome, the strains are rather diverse, therefore the design of primer pairs, suitable for diagnostic purposes or real-time reactions seems to be difficult. These results as well as the high diversity of the Hungarian strains shows, that the evolution of HEV is intensive.

The results of the investigation of both the partial and complete genome sequences shows, the HEV strains with animal origin are genetically related to strains, which were detected in human. In addition, on the phylogenetic trees, the distinct "Hungarian" branches in the subgroups consist of viruses with both animal and human origin. However epizootiological connections were not proved, these results support the theories suspecting the zoonotic potential of HEV.

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PHYLOGENETIC ANALYSIS OF AVIAN HEPATITIS E VIRUS ISOLATES FROM EUROPEAN AND AUSTRALIAN CHICKEN FLOCKS SUPPORTS THE EXISTENCE OF THREE GENOTYPES WITHIN AVIAN HEV

Ivana BILIC, Ana BASIC, Irina PROKOFIEVA, Michael HESS

Clinic for Avian, Reptile and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria

Keywords: big liver and spleen disease, capsid, helicase, hepatitis E virus, hepatitis-splenomegaly syndrome

Introduction and objectives

Investigations about the presence of avian HEV in chicken flocks are very limited and restricted to a few reports from Australia and the USA (Payne et al., 1999; Huang et al., 2002; Sun et al., 2004). Recently, two independent outbreaks of the big liver and spleen disease (BLSD) in broiler breeders in Europe were reported and wide spreading of avian HEV in chickens from Spain was also suggested (Peralta et al., 2008). In addition, recently was proposed that avian HEVs represent a separate genus within the family Hepeviridae and can be separated into three different genotypes (Bilic et al., 2008). We were interested in determining whether partial helicase and capsid gene sequences display relationships among avian HEV isolates similar to those that have been previously demonstrated by analysis of complete genome sequences (Bilic et al., 2008).

Materials and methods

Most samples have been prepared from birds demonstrating clinical and/or pathomorphological symptoms of HS syndrome or BLSD. However, some samples have been prepared from clinically healthy birds and the sampling was conducted as part of a routine survey. Using PCR for the regions of helicase and capsid genes, the presence of avian hepatitis E virus (avian HEV) was determined in European and Australian chicken flocks. A total of 27 virus isolates from 9 countries were used to determine the phylogenetic relationship following helicase and capsid PCRs and nucleic acid sequencing. For comparison, helicase and capsid sequences of completely sequenced avian HEVs from Europe, Australia and the USA were used. In addition, available helicase and capsid sequences of other avian HEVs and four mammalian HEVs were also used.

Results

In this study, the samples positive for presence of avian HEV RNA originated from 8 European countries and different regions of Australia. That suggested wide spreading of the virus within Europe. Sequence analyses of studied isolates revealed that European and Australian isolates of avian HEV are genetically heterogenic. Three genotypes within the avian HEV were revealed. In general, each genotype (1 to 3) corresponded to a specific geographical region: Australia, USA and Europe, respectively (Fig 1). However, several North American isolates clustered together with European isolates. In this study, phylogenetic analyses based both on helicase and capsid regions did not show any clustering of isolates according to whether the viruses were isolated from broiler breeder hens or commercial egg laying hens. All tested isolates consistently segregated into the same clusters when analyses were based on helicase or capsid sequences.

Discussion and conclusions

This is the first report describing the presence of avian HEV RNA in chickens from 8 European countries demonstrating its wide distribution in this region. In addition, genetic heterogeneity of avian HEV isolates from Europe and Australia was noted. The presented sequence analysis data, from both layers and broilers together with clinical observations are of importance to understand the epidemiology of avian HEV infections in chickens and to improve molecular diagnostic techniques. Phylogenetic analyses based on the partial capsid and helicase gene sequences confirmed the distant relationship of avian HEV to mammalian HEV, which is an important aspect with regard to zoonosis. In addition, they confirm that the avian HEVs can be separated in three different genotypes. Furthermore, the results of both analyses were in accordance. Finally, we suggest that the partial capsid and helicase gene sequences could be used for phylogenetic analysis of avian HEVs providing a useful molecular epidemiological tool.

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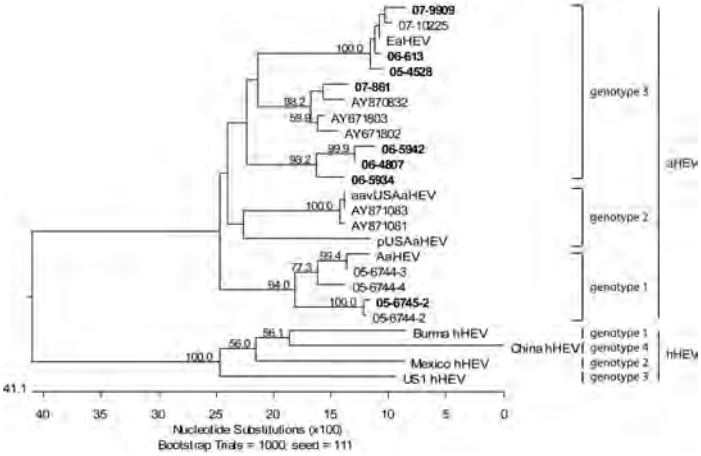

Figure 1: Phylogenetic tree based on the sequences of the helicase gene of HEV isolates over a 130 bp region. Scale bar representing the number of character state changes is shown. Branch lengths are proportional to the number of character state changes. Bootstrap values are indicated for the major nodes. Sequences from this study are labelled in bold.

DISCOVERY OF NEW ENTERIC VIRUSES, NOROVIRUSES, IN DOGS

Vito MARTELLA[1], Arianna RADOGNA[1], Eleonora LORUSSO[1], Paschalina MOSCHIDOU[1], Krisztián BÁNYAI[2], Gabriella ELIA[1], Decaro NICOLA[1], Costantina DESARIO[1], Canio BUONAVOGLIA[1]

[1]Department of Veterinary Public Health, University of Bari, Valenzano (Bari), Italy;
[2]Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary

Keywords: enteritis, norovirus, zoonosis

Introduction and objectives

The Caliciviridae are small non-enveloped viruses of approximately 35 nm in diameter with single-stranded, positive-polarity RNA genomes of 7.4–8.3 kb (1). The family includes the genera *Vesivirus*, *Lagovirus*, *Norovirus*, *Sapovirus* and unassigned recently identified bovine and simian caliciviruses. Noroviruses (NoV) and sapoviruses are considered important gastroenteric pathogens in humans of all age groups. The viruses are highly contagious and are transmitted by direct contact or by contaminated water and food (2). Some evidence suggests a zoonotic potential of animal enteric caliciviruses, but this issue is still controversial. NoVs have been identified in pigs, cows and mice. Recently, we identified a mixed infection by calicivirus and parvovirus type-2a in a dog with gastro-enteritis (4). The calicivirus strain, Bari/170/07-4/ITA, was found to resemble a lion NoV strain, Pistoia/387/06/ITA, previously detected in a captive lion cub with enteritis (3), and to a lesser extent Alphatron-like human NoVs (GIV genogroup). Identity between the canine and lion NoVs was 90.1% aa, while identity to GIV human NoVs was 68.2%-69.4 aa and to non-GIV NoVs was < 53.3%. Accordingly, the animal GIV strains were classified as a distinct genotype (GIV.2) from the human GIV NoVs (GIV.1). In order to assess the epidemiology of NoVs in dogs, a surveillance study was initiated by implementing, with NoV-specific assays, the diagnostic algorithms of all the cases of gastro-enteric disease reported to our laboratories during the year 2007.

Materials and methods

Collection of faecal samples and screening for canine viral pathogens and noroviruses. A total of 183 stool samples were collected between January and December 2007 from young dogs (aged 1 to 6 months) with signs of mild to severe gastro-enteritis. All faecal samples were screened for the presence of common canine viral pathogens by either gel-based PCR or quantitative PCR and RT-PCR (qPCR and qRT-PCR). The RNA extracts were screened for NoV using a broadly reactive primer pair, p289-p290, and NoV-specific primers JV12Y-JV13I, that target highly conserved motifs "DYSKWDST" and "YGDD" of the RdRp region.

Sequence analysis. The amplicons were excised from the gel, purified and sequenced. In order to determine the sequence and genomic organization of the NoV strains Bari/91/07/ITA, a 3.5 kb region at the 3' end of the genome was amplified by RT-PCR using primers p290 and a poly-T primer. The amplicon was purified, cloned and sequenced.

Analysis of the capsid protein VP1 of strain Bari/91/07/ITA. Pair-wise identity in the full-length VP1 of strain Ca/Bari/91/07/ITA to 171 NoV strains was determined using multiple alignments following the outline of Zheng et al., (5). The values were calculated by the uncorrected distance method using a 172-sequence alignment without removing the gaps. The sequence (3510 nt) from the 3' end of ORF1 to the poly-A tail of the canine NoV Bari/91/07-4/ITA was made available in Genbank under accession number FJ875027.

Results

Screening for canine viral pathogens and for noroviruses. Out of 183 samples, 72 (39.3%) contained CPV-2, either alone (51=28%) or in mixed infection (19=11.3%). Thirty-five samples (19.1%) contained CCoV RNA, alone (17=9.2%) or in conjunction (18=9.9%) with other viruses. CAV-2, CrCoV, and CDV were detected sporadically in 2/183 (1%), 1/183 (0.5%) and 1/183 (0.5%), respectively, while rotaviruses were not detected. Along with strain Bari/170/07-4/ITA, an additional 3 samples (445/07-A, 445-07-B and 91/07) were found to contain NoV RNA. Three of those four samples also contained CPV-2, but did not contain other viral pathogens.

Sequence analysis of the canine NoV strains. Sequence analysis of the 315 bp fragments of the RdRp region the strains 445/07-A, 445-07-B and Bari/91/07/ITA revealed the highly conserved calicivirus motive GLPSG. By sequence comparison, in the short RdRp fragment the viruses 445/07-A, 445-07-B and Bari/91/07/ITA were closely related to the canine NoV strain GIV.2/Bari/170/07-4/ITA (91.7-94.0 % nt). The 3.5 kb fragment of the genome of strain Bari/91/07/ITA (the 3' end of ORF1, the full-length ORF2, ORF3 and the non-coding region trough the poly-A tail), was determined. The 3' partial sequence of ORF1 spanned 808 nt and 268 aa at the COOH-terminus of the polymerase complex. By BLAST and FASTA analysis, the highest identity was found to the canine strain GIV.2/Bari/170/07-4/ITA (91.8 nt and 99.3 % aa) and to the lion NoV strain Pistoia/387/06/ITA (85.7% nt and 97.0% aa). There was a single nt overlap between ORF2 and ORF3; also, there was a 131-nt long non-translating region between ORF3 and the poly-A tail. ORF3 was 837 nt in length and encoded for a 279-aa polypeptide.

Analysis of the capsid protein VP1. The ORF2 was 1749 nt long and contained an ORF encoding a capsid protein with a predicted size of 582 aa. In the ORF2, by preliminary analysis with BLAST and FASTA, the highest sequence match (88.9% and 98.9% aa) was found to three short NoV sequences (281 nt in length) detected from oysters in Japan 2003-2005. These short sequences overlapped the 5' end of the ORF2. Identity to any other NoV sequence in this short fragment was <78.0% nt, with the best match being to strain Chiba/040502/04/Jp, and <85% aa. Pair-wise aa distances were calculated using the full-length VP1. In this analysis, the VP1 protein of canine NoV Bari/91/07/ITA was more related (58.1% aa) to the human strain Chiba/040502/04/JAP, rather than to GIV human and animal NoVs (54.0-54.4% aa). The two viruses, Bari/91/07/ITA and Chiba/040502/04/JAP, displayed <54.7% and <53.9% aa identity, respectively, to any other NoV strain within genogroups I, II, III, IV and V. Based on widely accepted classification criteria, strains within the same genotype (or cluster) share > 85% aa identity, while strains of different genotypes within the same genogroup share 55-85% aa identity (5). Accordingly, the canine virus Bari/91/07/ITA and the human virus Chiba/040502/04/JAP are each the prototype of a novel genotype, likely within a novel NoV genogroup, tentatively proposed as GVI.

Discussion and conclusions

The findings of this study demonstrate that (i) NoVs circulate in dogs, (ii) that canine NoVs are genetically heterogeneous, with more genogroups/genotypes, although only a few NoV strains have been characterised in detail thus far, and (iii) that humans may be exposed to canine-like NoVs via certain transmission routes, e.g., by consumption of raw bivalve molluscs, since the very 5' end of ORF2 of the canine virus Bari/91/07/ITA matched short noroviral sequences (88.9% nt and 98.9% aa) identified from oysters in Japan, thus indicating that similar viruses may be common environmental contaminants.

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DETECTION OF CONTEMPORANEOUS HUMAN AND BOVINE NOROVIRUSES IN BELGIUM

Alexandra SCIPIONI[1], Axel MAUROY[1], Elisabeth MATHIJS[1, 2], Dominique ZIANT[1], Georges DAUBE[2], Etienne THIRY[1]

[1]University of Liège, Department of Infectious and Parasitic Diseases, Virology and Animal Viral Diseases, Liège, Belgium;
[2]University of Liège, Department of Food Sciences, Microbiology, Liège, Belgium

Keywords: bovine, genotyping, norovirus, recombinant, zoonosis

Introduction and objectives

Noroviruses (NoVs) belong to the family *Caliciviridae*. They have emerged as a common cause of infectious gastroenteritis in all age groups in humans, especially in restaurants and institutions such as nursing homes and hospitals and are the main cause of foodborne gastroenteritis. In the 1980s and the 1990s they were described in bovine and porcine species, respectively (1). To date, at least five genogroups have been described: GI and GII involving the majority of human viruses; GIII, the bovine NoVs; GIV, at least two human NoVs (strains Alphatron and Fort Lauderdale) and GV, the murine NoV. The hypothetical mechanisms behind the periodic emergence of epidemic strains range from a large-scale person to person (eventually foodborne) transmission of a single "new" strain to an introduction from a non-human reservoir. This particular hypothesis can be substantiated by the detection of partial human NoV sequences in stool samples from cattle and pigs in Canada. The accumulation of NoV nucleotide sequences is therefore crucial in the study of the phylogenetic and molecular relationships between animal and human NoVs. Moreover, hypothetical recombinant strains could be detected through a divergent clustering by analyzing different parts of the NoV.

The aim of this study was to detect and to genetically characterize NoVs in fecal samples from both contemporaneous humans and cattle using RT-PCR assays with different sets of primers. Sequences from different regions of the NoV genome were determined in order to investigate the genetic variation of these strains and to allow comparison with prototype strains of the different NoV genogroups and genotypes.

Materials and methods

Stool specimens from cattle were collected by the regional animal diagnostic laboratories of "ARSIA" (Association Régionale de Santé et d'Identification Animales), in Belgium over a two year period (2002 – 2003): samples were collected from cattle of all age groups (317 samples) and two samples detected in 2007 were added. In the same period, 603 human samples were collected from the medical microbiology and virology service of the University Hospital of Liege, Sart-Tilman. Other human samples from outbreaks were provided by the Institute for Public Health in Brussels and the Virology Laboratory of the St Luc University Hospital for the period 2006-2007. They were all stored at 4 °C before analysis and then at -80 °C.

RNA was extracted with the QIAamp viral RNA mini kit (Qiagen, Leusden, the Netherlands) and RT-PCR were conducted with the Access RT-PCR system kit (Promega, Leiden, the Netherlands), with primer pairs JV12-13, CCV3-CCV4, CBECUF-R (2 ; 3 ; 4). Purified RT-PCR products were sequenced in both directions with the same primers as for RT-PCR. For long fragments, cloning in Topo zero blunt (Invitrogen, Merelbeke, Belgium) with Top10 bacteria was carried out according to the manufacturer's instructions and a consensus sequence was determined for at least 3 clones.

The nucleotide sequences were compared with international databases using the BLAST program. Sequences were aligned using the ClustalW program with its default settings in the Bioedit program. Phylogenetic and molecular evolutionary analyses were conducted using MEGA, Version 4.

Results

Thirty three and twenty eight sequences from human samples were confirmed positive for NoV using JV12-JV13. Partial NoV sequences belonging to GI.2, GI.5, GII.2, GII.3, GII.4 (1996-2002-2004-2006-2006a-2006b), GII.6, GII.b were detected. The predominance of GII.4 NoVs was noticed, since 88.6% (31/35) of positive samples contained this genotype. Some strains showed a different genotyping depending on the investigated part of the genome. The sequencing of a 1.1 kb product beginning in the polymerase coding region and ending in the capsid coding region (covering the recombination hot spot identified in NoVs) revealed that H007 is in fact a coinfection of a GII.2 and a GII.6 NoV. Strains H325 (GIIb/GII.3) and H501 (GI.2/GI.8) are natural recombinants. A Simplot analysis indicated the recombination point to be at the ORF1-ORF2 junction.

Six samples from cattle showed RT-PCR products of the expected size using JV12-JV13 primers, eight using CBECUF-R primers and ten using CCV3-CCV4 primers. Phylogenetic studies using partial RNA polymerase and partial capsid coding regions showed that the detected bovine NoVs are distinct from representatives of GI and GII NoV. They are all closely related and cluster in the genogroup III with sequence identity on amino acid between these strains and the Newbury2 agent ranging between 95.9% and 99.6%. No evidence of recombination was identified in bovine NoVs.

Discussion and conclusions

Contemporaneous human and bovine NoVs were detected in Belgium and their clustering following genetic sequences showed that human NoVs cluster with GI and GII representatives, while bovine NoVs cluster with the GIII. These results support the idea of a NoV species specificity rather than a zoonotic transmission or an animal reservoir. The identification of recombinant strains evidences the natural phenomenon of recombination in the genus *Norovirus*.

Fifteen and thirty five samples were confirmed as positive by sequencing for bovine and human norovirus respectively. The choice of primer pairs used in this study was at first deliberately a primer pair designed to detect human NoV (JV12-JV13), with the objective of finding, in bovines, caliciviruses closely related to human NoVs. Additional primer pairs were used to increase the chance of bovine NoV detection.

The genotype II.4 is recognized as the predominant human genotype worldwide, and data from this study support this, with the finding of 88.6% of GII.4 among the human detected NoV strains.

Two natural recombinants were detected, one intra-genogroup GI, and one intra-genogroup GII. The majority of the previously identified recombinants show a recombination hot spot located at the ORF1-ORF 2 junction, which could correspond to the observed results in this study. A multiple infection was detected in another sample, H007. Indeed, recombination could occur with a high probability in the case of multiple infections.

Phylogenetical trees showed that bovine NoVs detected in this study are closely related to each other and that they cluster within the GIII.2 genoroup. This observation supports the idea that bovine NoVs are specific to this species. Genogroup III.1 bovine NoV was not detected in this study. This study confirms the predominance of GIII.2 strains.

There is no evidence of strains circulating in both humans and cattle or of cross contamination. Nevertheless, the possibility of a potential zoonotic infection or a cattle reservoir for human infection cannot be excluded, because of the close relationship with human NoV sequences and the proximity of the human population and animals in a country like Belgium.

Furthermore, on the one hand, it has been shown to be possible for pigs to be experimentally infected with human GII NoV and, on the other hand, an intergenogroup recombinant has been previously described. This highlights the risk of recombination between human and animal NoVs, leading to new NoV strains, which could infect both humans and animals. Therefore, it would be advisable to continue the study of NoVs in animals and humans and to develop methods for the detection of such interspecies transmission at an early stage.

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DIFFERENT MECHANISMS CONTRIBUTE TO RNA GENOME REPAIR IN THE 5´ NONTRANSLATED REGION OF BOVINE VIRAL DIARRHEA VIRUS

Stefanie GILGENBACH[1], Simone PREIS[2], Paul BECHER[1]

[1]Institute of Virology, University of Veterinary Medicine, Hannover, Germany;
[2]Institute of Virology, Justus Liebig-University, Gießen, Germany

Introduction and objectives

A fundamental feature of RNA viruses is represented by their high genetic variability. Point mutations during RNA synthesis and RNA recombination are the main driving forces for virus evolution and may also be involved in viral RNA genome repair by eliminating disadvantageous and lethal mutations and thereby contribute to genome conservation. The aim of the current study was to investigate the mechanisms of RNA genome repair in the 5´ nontranslated region (NTR) of bovine viral diarrhea virus (BVDV). BVDV is the type species of the genus *Pestivirus* within the family *Flaviviridae*. Pestiviruses represent a group of highly variable RNA viruses which cause economically important livestock diseases such as bovine viral diarrhea/ mucosal disease and classical swine fever. The pestivirus genome consists of a positive-stranded nonpolyadenylated RNA molecule of approximately 12.3 kb containing one large open reading frame (ORF) flanked by 5´ and 3´ nontranslated regions.

Material and methods

Construction of cDNA clones was based on the cytopathogenic BVDV strain CP7 and the authentic, infectious full-length cDNA clone pCP7-388 described previously (Meyers et al., 1996; Becher et al., 2000; Pankraz et al., 2005). To study RNA genome repair synthetic genome-like RNAs with various deletions at the 5´ end and short 5´ fragments of the BVDV RNA genome were generated by *in vitro* transcription using SP6 RNA polymerase. After DNase I digestion these RNAs were transfected or cotransfected into bovine MDBK cells by electroporation (Becher et al., 2000; Gallei et al., 2004). The cells were then seeded on 24 well-plates and microscopically examined from day 3 to 5 post transfection to determine the number of wells with a cytopathic effect (cpe). Finally, total cellular RNA was prepared and used for RT-PCR analyses in order to determine the 5´ and 3´ terminal sequences of the genomes of the emerged viruses. The respective methods have been previously described (Becher et al., 2000). Prior to nucleotide sequencing the PCR products were cloned into a plasmid vector.

Results

The specific infectivity for some of these genome-like RNAs with various deletions at the 5´ end was nearby the detection limit (<10 PFU/µg RNA), while for others no infectious virus could be recovered. Analysis of 150 independently emerged viruses by RT-PCR and nucleotide sequencing led to detection of various genetic changes resulting from different repair mechanisms:

I) Point mutations, deletions and duplications of 1 to 6 nucleotides at or near the 5´ end of the mutated genomes.
II) Nonhomologous RNA recombination with various cellular RNA sequences. Using the Blast option of the NCBI molecular database most sequences could be assigned to bovine mRNAs.
III) Nonhomologous RNA recombination with viral coding sequences (e.g. E2, NS2).
IV) Nonhomologous and homologous RNA recombination with cotransfected short 5´ NTR fragments.

Furthermore, viral growth properties (plaque morphology and growth kinetics) and viral RNA synthesis were analysed for selected 5´ mutants. Most of them showed slightly delayed growth rates. All investigated mutants produced smaller plaques and less amounts of viral RNA than parental BVDV CP7.

Discussion and conclusion

To study the molecular basis of viral RNA genome repair, we generated synthetic genome-like RNAs of BVDV strain CP7 with various deletions at the 5´ end and transfected them into MDBK cells. Nucleotide sequence analysis of 150 emerged viruses demonstrated that the infectivity of the mutated viral RNA genomes was restored by at least four different repair mechanisms (see above). Taken together, the results of our study provide clear evidence that in addition to point mutations, deletions and additions of single nucleotides, RNA recombination with both cellular and viral RNAs significantly contributes to repair of RNA genomes. Irrespective of the mode of repair a 5´-GUAU motif that has been previously suggested to represent an important signal for viral replication (Frolov et al., 1998; Becher et al., 2000) was restored in each of the emerged viral genomes.

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CHEMOKINE TRANSCRIPTION IS DIFFERENTLY MODULATED IN RAINBOW TROUT (*Oncorhynchus mykiss*) INFECTED WITH VIRAL HAEMORRAGHIC SEPTICAEMIA VIRUS (VHSV) AND INFECTIOUS PANCREATIC VIRUS (IPNV)

Jana MONTERO, **Elena CHAVES-POZO**, **Alberto CUESTA**, **Carolina TAFALLA**

Centro de Investigación en Sanidad Animal, CISA-INIA, Madrid, Spain

Keywords: chemokines, infectious pancreatic necrosis virus (IPNV), poly I:C, rainbow trout, viral hemorraghic septicaemia virus (VHSV),

Introduction and objectives

Chemokines constitute a family of cytokines with chemoattractant properties for immune cells, that also exert immunomodulatory actions, thus modulating the immune functions of their target cells. Nowadays, twenty four chemokines have been identified in rainbow trout (*Oncorhynchus mykiss*), however, even though their sequences have been reported, their biological role has not been fully elucidated. Moreover, the role that these chemokines have on the antiviral response in fish has been poorly studied. In this work, we have determined the levels of expression of several of these rainbow trout chemokines in head kidney and spleen, during the course of a viral infection using viral haemorraghic septicaemia virus (VHSV) and infectious pancreatic necrosis virus (IPNV), and compared them to the levels induced by poly I:C. We also determined the effects that the two viruses and poly I:C provoked on the levels of expression of theses chemokines in vitro in head kidney leukocytes. A preliminary study to elucidate the effect on interferon (IFN) of three of these virus-induced chemokines is also reported using eukaryotic expression plasmids.

Materials and methods

Rainbow trout were obtained from El Molino (Madrid, Spain), located in a VHSV and IPNV-free zone. Prior to the experiments, fish were acclimatised to the laboratory conditions for 2 weeks.

VHSV (0771 strain) and IPNV (Sp strain) propagated in the RTG-2 cell line and tritated as previously described (Reed and Muench, l988) were used for the experiments

In order to study the chemokine expression *in vivo*, rainbow trout were divided in five groups of 20 fish each. Groups were injected intraperitoneally with either poly I:C and viral solution, using PBS as control group for the poly I:C-treated fish, while the control group for the virus infected fish was the culture medium. At days 1, 3, 7 and 10 post-injection, five trout from each group were sacrificed by overexposure to MS-222, and head kidney and spleen were removed for RNA extraction.

Briefly, the anterior kidney was removed aseptically and leukocytes were isolated by using 51% Percoll density gradients; following the method previously described (Graham and Secombes, 1988). Cells in a concentration of $1x10^6$ cells ml^{-1} were disposed in a 24-well plate and after 3 hours were exposed to poly I:C or infected with VHSV or IPNV (m.o.i. of 0.1). The RNA of the cell was extracted after a further of 24h of incubation.

Total RNA was extracted from organs or cells using Trizol (Invitrogen) following the manufacturer´s instructions. RNA from the different individuals in each group were pooled, treated with DNAse I, and one µg of this pooled RNA was used to obtain cDNA using the superscript III reverse transcriptase (Invitrogen).

In order to evaluate the transcription of chemokines studied, real-time PCR was performed using SYBR Green PCR Core Reagents (Applied Biosystems). For each mRNA, gene expression was corrected by the EF-1α expression in each sample as previously described (Cuesta and Tafalla, 2009).

PCR products encoding the entire open reading frame of CK5B, CK6 and CK7A were cloned into the expression vector pcDNA3.1/V5-His-TOPO according to manufacturer´s instructions (Invitrogen). The resulting constructs were designated as pCK5B, pCK6 and pCK7A. A religated empty pcDNA3.1/V5-His-TOPO plasmid (pcDNA) was used as a negative control.

In order to determine the effect that the intramuscular injection of the different chemokine plasmids produced, 16 trout in each group were intramusculary injected with either pCK5B, pCK6 or pCK7A (1 µg in 100 µl of PBS per fish); the same amount of the empty construct used as negative control (pcDNA); or with the same volume of PBS.

At days 1, 2, 5 and 7 post-injection, four trout from each group were sacrificed by overexposure to MS-222, and head kidney and spleen removed for RNA extraction. The levels of expression of type I and II IFN and Mx were evaluated as previously described for the chemokines.

Results

In the case of the spleen, poly I:C and VHSV up-regulated the transcription of CXCd and γIP CXC chemokines and CK3, CK5 and CK12 CC chemokines. VHSV but not poly I:C also induced CK6. IPNV however, only had a significant stimulatory effect on CK6 and CK7A. In the head kidney, VHSV also increased the expression of CXCd, γIP, CK3 and CK12 chemokines, having no significant effect in the levels of expression of CK5B or CK6 in this organ, in which the response to poly I:C was similar to that of the spleen. IPNV again showed a distinct pattern, and only increased the expression of CK1, CK5B and CK7A. In the case of head kidney leukocyte cultures, poly I:C induced the transcription of CK1, CK5B and CK12, while VHSV induced γIP, CK1, CK3, CK5B, CK6 and CK12 as previously observed in spleen. IPNV in vitro increased CK1, CK5B, CK9 and CK12 mRNA levels.

The three chemokine expression plasmids constructed for CK5B, CK6 and CK7A induced an increased type I IFN expression in the head kidney, although the greater effects were observed in response to pCK6 at day 2. In the spleen, however, only pCK5B enhanced the levels of IFN I, and pCK6 and pCK7A even significantly down-modulated its expression at day 2. In the case of Mx, pCK6 and pCK7A induced its expression both in the head kidney and spleen.

Regarding type II IFN, IFN-γ transcription was enhanced in head kidney with pCK5B, pCK6 and pCK7A and with pCK6 and pCK7A in spleen, although as it occurred with the type I IFN genes, a much stronger response was observed in response to the pCK6 plasmid in both organs.

Discussion and conclusions

Our results show that VHSV and IPNV induce a different profile of chemokine expression. Moreover, each virus induces a different profile in the different organs studied. As more information becomes available concerning the immune role and target cells that these chemokines have on rainbow trout, we would be able to better interpret the importance of these differences in the pathogenicity of these two viruses.

Preliminary studies using expression plasmids for three of these virus-induced chemokines grouped into what phylogenetically has been catalogued as “inducible” CC chemokines show a clear effect on type I and type II IFN production.

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IN VITRO ONCOLYTIC CAPACITIES OF TWO MYXOMA VIRUS STRAINS

Magalie GRETILLAT[1, 2], Philippe ERBS[3], Jacqueline GELFI[1, 2], Christelle CAMUS[1, 2], Stéphane BERTAGNOLI[1, 2]

[1]*INRA, UMR 1225, F-31076 Toulouse, France*
[2]*Université de Toulouse; ENVT ; F-31076 Toulouse, France*
[3]*Transgene SA, 11 rue de Molsheim, Strasbourg Cedex, France*

Keywords: human tumoral cells, myxoma virus strains, oncolytic vector, virotherapy

Introduction and objectives

Virotherapy exploits the use of natural and engineered Oncolytic Viruses (OV) to selectively kill tumor cells. Tumor-selective OVs are intended to replicate, propagate and spread specifically in tumor cells leading to their destruction, while not affecting normal cells.

Myxoma virus (MYXV), agent of myxomatosis in European rabbits, has a very narrow host range. However, MYXV productively infects a variety of human cancer cell lines (3), is oncolytic for human pancreatic adenocarcinoma cell lines (4), and has a significant antitumor activity against experimental human gliomas and rhabdoid tumors (2, 5). In some cases, oncolytic capacities of OV can be optimized by inactivation of viral genes that are needed for replication in normal cells but not in tumor cells. The vaccine strain of MYXV (SG33-MYXV) is a cell culture-attenuated strain with a large genome deletion encompassing several genes encoding for immunomodulatory and anti-apoptosis factor.

In order to use MYXV as OV against colorectal, pancreatic and ovarian cancers, we investigated viral replication under single and multistep conditions and compared oncolytic capacities of wild type strain (T1-MYXV) and vaccine strain (SG-MYXV) of MYXV in several human tumor cell lines *in vitro.*

Materials and methods

Cells: permissive control RK-13 cells (rabbit kidney cells, n°ATCC CCL-37); human tumor cell lines from the NCI-60 reference collection: OV-CAR-5 and SK-OV-3 (human ovarian adenocarcinoma cells, n°ATCC HTB-77); PANC-1 and Capan-2 (human pancreatic adenocarcinoma cells, n°ATCC CRL-1469 and HTB-80); HCT-116, Caco-2 (human colorectal adenocarcinoma cells, n°ATCC CCL-247 and HTB-37).

Virus: T1-MYXV, wild type strain isolated in Toulouse in 1952; SG-MYXV, cell culture-attenuated vaccine strain with a large genome deletion encompassing several immunomodulatory genes.

Viral replication: cells infected with MOI of 3 under synchronized conditions (adsorption phase at 4°C) were harvested at 0h, 4h, 12h, 24h and 48h post infection. The viral production was determined by titration on RK-13 cells.

Cell viability: cell viability was determined with neutral red assay five days post infection (when complete lysis of permissive control RK-13 was observed) at MOI of 5.

Cell sensitivity to 5-FC: cells were infected with Modified Vaccinia virus (MVA) and MYXV expressing FCU1 at respective MOI of 0.01 and 0.1, and incubated with various concentration of 5-FC. After 7 days of incubation, viable cells were counted by trypan blue exclusion.

Results

Replication of T1-MYXV and SG-MYXV in synchronized infections. Compared to T1-MYXV, the SG-MYXV strain is always producing higher yield of viral progeny in every cell lines after 48 h of infection at MOI of 5. In three out of six cell lines, the increase in viral yield is even almost the same than in permissive RK-13 cells. The higher adsorption rate of SG-MYXV can account for this difference in global virus yield, as viral titer observed after synchronized adsorption is always superior with SG-MYXV infection compared to T1-MYXV.

In vitro sensitivity of human tumor cells to MYXV strains. Although cell survival doesn't seem to be impaired in Capan-2 and OV-CAR-5 cells at MOI of 5 whatever MYXV strain used, it appears that percentage of tumor cell survival is reduced in the four other tumor cell lines (HCT-116, Caco-2, PANC-1 and SK-OV-3). Moreover, a significant decrease in cell survival can be observed after infection with SG-MYXV strain compared to T1-MYXV type strain infection (figure 1). We also explored the behavior of SG-MYXV at lower MOI in sensitive HCT-116, PANC-1, Caco-2 and SK-OV-3 cells and found, as previously shown in PANC-1 cells (4), that effective oncolysis of human tumor cells by MYXV occurs in dose dependant manner.

In vitro sensitivity of human tumor cells to armed SG-MYXV. In order to counterbalance this loss of activity at lower activity and in non sensitive cells, the SG-MYXV strain was armed with the highly potent *fcu1* suicide gene (1). All tumor cell lines infected with SG-FCU1 at low MOI (0.1) become sensitive to 5-FC after an incubation of 6 days in the same extent than cell lines infected with MVA-FCU1 (MOI 0.01) (illustrated in Capan-2, figure 2). Chemotherapy mediated by *fcu1* suicide gene seems to be able to counterbalance the loss of activity of SG-MYXV at low MOI in sensitive cells and the lack of oncolytic activity in non sensitive cells.

Fig 1. Cell survival after infection with T1-MYXV and SG-MYXV at MOI of 5

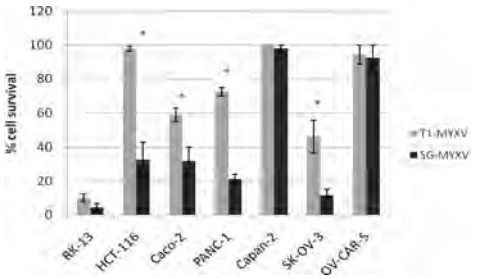
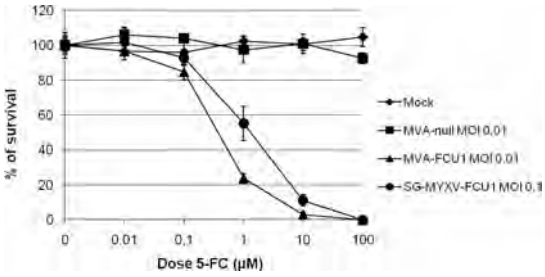


Fig 2. Cell sensitivity of Capan-2 cells to 5-FC after infection with SG-MYXV-FCU1



Discussion and conclusions

Our study revealed the interesting capacities of the vaccine strain of MYXV in terms of replication and cell killing, compared to wild type strain, in a panel of tumor cells from three different tissue origins.

The numerous genomic modifications that have been observed in SG-MYXV sequence (analysis still ongoing) could explain this superiority in tumoral cells. For example, a five amino acids (aa) deletion was found in the MYXV M083L aa sequence that could explain part of the higher adsorption rate of SG-MYXV, as the vaccinia virus (VACV) homolog, membrane D8L protein, has been reported to play a role in virus attachment to cell surface chondroitin sulfate. Then, the fact that four genes encoding proteins known to counterpart cellular antiviral defense are absent in the SG-MYXV sequence may be related to phenotypic difference observed, as poxvirus tropism at cellular level is rather regulated by intracellular events.

We have also demonstrated that the limited viral activity of oncolytic vector, at low MOI or in poorly sensitive cells, could be counterbalance by specific molecular chemotherapy. Here, arming the SG-MYXV strain with the highly potent *fcu1* suicide gene have restored cell killing in non sensitive cells and at low MOI of oncolytic vector.

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VIRUS-HOST ADAPTATION: WHATS NEW ON MYXOMATOSIS?

Alexandra MULLER[1, 2], Eliane SILVA[1, 2], Joana ABRANTES[3, 4, 8], Pedro J. ESTEVES[3, 5], Paula G. FERREIRA[6], Júlio C. CARVALHEIRA[1, 3], Norbert NOWOTNY[7], Gertrude THOMPSON[1, 2]

[1]*Institute of Biomedical Science Abel Salazar (ICBAS), Department of Veterinary Clinics, University of Porto, Porto, Portugal;*
[2]*Multidisciplinary Unit of Biomedical Investigation (UMIB), University of Porto, Porto, Portugal;*
[3]*Centre of Investigation for Biodiversity and Genetic Resources (CIBIO), University of Porto, Vairão, Portugal;*
[4]*Department of Zoology and Antropology, Faculty of Sciences, University of Porto, Porto, Portugal;*
[5]*Centro de Investigação em Tecnológias da Saúde (CITS), CESPU, Portugal;*
[6]*Institute of Biomedical Science Abel Salazar (ICBAS), Department of Anatomy, University of Porto, Porto, Portugal;*
[7]*Zoonoses and Emerging Infections Group, Clinical Virology, Department of Pathobiology, University of Veterinary Medicine, Vienna, Austria;*
[8]*INSERM, U892, Université de Nantes, Nantes, France*

Keywords: co-evolution, European rabbit, field strains, myxoma virus, sequence analysis

Introduction and objectives
Myxoma virus infection in its natural host, rabbits of the genus Sylvilagus in South America, causes benign disease. However, a severe pandemic with high mortality rates was observed after its introduction in Australian and European wild rabbit populations (genus Oryctolagus) in the 1950s. Since, myxomatosis has been among the best studied examples of host-pathogen co-evolution. However, information on viral genetic changes related is scarce. Our objectives were to study selected genomic regions of recent myxoma virus field isolates and to evaluate the suitability of these as molecular marker for virus evolution within the European rabbit.

Material and Methods
Nine field strains were obtained from European rabbits displaying signs of myxomatosis. They were collected between 2004 and 2007 in Portugal. DNA was extracted from the second passage of the field isolates in RK-13 cells. Nine genomic regions spanning 11 genes of the myxoma viruses were directly sequenced and compared with the original virulent strain "Lausanne" and its attenuated field derivative strain "6918". The attenuated vaccine strain RIAM was used as reference.

Results
The field strains displayed a maximum of three (strains C43, C95) and a minimum of one nucleotide substitutions (strains CD01, CD05). These were distributed through all analysed coding regions, except gene M022L (major envelope protein), where all strains were identical to "Lausanne" and "6918". Two new single nucleotide insertions were observed in some of the field strains: within the intergenic region M014L/M015L and within gene M009L, leading to a frameshift. These insertions were located after homopolymeric regions.

Discussion and Conclusions
Our results support a high degree of genetic stability of myxoma virus over the past five decades. None of the analysed genome regions by its own seems sufficient for the genetic characterization of field strains.

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MYXOMA VIRUS AS VACCINE VECTOR FOR NEW VACCINATION STRATEGY AGAINST BLUETONGUE

Sokunthea TOP[1], Béatrice PIGNOLET[1], Eliane FOULON[1], Martine DEPLANCHE[1], Stéphane BERTAGNOLI[1], Gilles FOUCRAS[1], Gilles MEYER[1]

[1]INRA, UMR 1225, F-31076 Toulouse, France;
Université de Toulouse; ENVT ; UMR 1225; F-31076 Toulouse, France

Keywords: bluetongue virus, bone marrow-derived dendritics cells, myxoma virus, ruminants, vaccine

Introduction and objectives

Poxviridae, a family of large DNA viruses, represent attractive tools for vaccine vector development against a broad spectrum of pathogens. This is particularly due to their capacity to induce a strong immune response, to integrate large inserts of foreign DNA, and their stability. Among poxviruses, *Myxoma virus* (MYXV), a virus which specifically infects *Leporidae* and causes a lethal disease called myxomatosis in European rabbits, is an interesting candidate for vaccine development. Recombinants MYXV were shown to be efficient for vaccination in rabbit [1], and cat [3]. Its potential as vaccine vector in non-leporide species was investigated in cattle and sheep in order to produce new vaccine against bluetongue. Bluetongue is an infectious arthropod-borne viral disease affecting domestic, wild ruminants and camelids caused by Bluetongue Virus (BTV). BTV is a double stranded RNA virus (genus *Orbivirus*), divided in 24 serotypes without cross protection between them. With the aim to test the efficacy of MYXV vector in sheep, we have first characterized MYXV infection in antigen-presenting cells. Then, *in vivo* infection with MYXV-VP60, a recombinant virus encoding VP60 (capsid protein of Rabbit Haemorrhagic Disease Virus), allowed us to evaluate the vector spread in sheep and its capacity to induce an immune response against specific antigen. Finally, immunisation with recombinant MYXV expressing bluetongue proteins, VP2, or VP2 and VP5 of BTV8 was carried out, and protection against a viral challenge was investigated.

Materials and methods

Cells, viruses: Bone marrow-derived dendritics cells (BM-DC) are harvested from tibia in rabbit and the sternum in sheep according to the protocols already described [2]. Rabbit kidney cells (RK13) were grown in Dulbecco's minimum essential medium supplemented with penicillin, streptomycin and 10% new born calf serum.

The SG33 MYXV attenuated strain [5] was used to construct recombinant viruses. SG33-GFP, SG33-VP60, SG33-VP2, SG33-VP2+VP5 are recombinant MYXV expressing green fluorescent protein, Rabbit Haemorrhagic Disease Virus capsid protein VP60, and synthetic structural protein VP2 only or with VP5 of Bluetongue serotype 8, respectively. All genes were inserted into the non essential MGF (myxoma growth factor) and M11L SG33 genes. SG33-Ø is a recombinant virus also deleted for MGF and M11L genes but without transgene of interest.

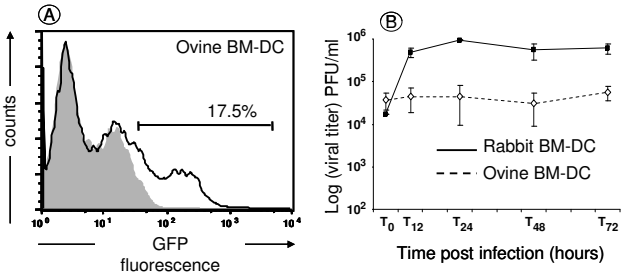
In vivo investigations: Two sheep were inoculated intradermally (i.d.) with various doses (10^5 to 10^7 pfu) of SG33-VP60 in multiple sites. Then, skin biopsies were taken at the injection sites from 0 to 72 h post-infection (p.i) for histological examination. In order to study humoral response, a group of sheep (n=10) was injected twice intradermally with 2.10^6 pfu of SG33-VP60 virus. Serum samples were taken at day 0, 21, 35 and 45 for each animal.

Three groups of sheep (n=5) were injected sous-cutaneously with 6.10^8 pfu of SG33-Ø, SG33-VP2, or SG33-VP2+VP5, at day 0, 20. A group of sheep (n=5), was vaccinated with the Merial alsap 8 vaccine (control group), and the last one represent sentinels group. All sheep were challenged with virulent BTV-8, 20 days after the last immunization. Clinicals observations were conducted daily after challenge during 25 days. At each time point, sheep rectal temperature was measured, blood samples were collected in order to check for seroconversion for the presence of BTV8 RNA by RT-PCR.

Results

Infection of antigen-presenting cells: In order to test the capacity of SG33 to infect antigen-presenting cells in sheep, BM-DC were infected with SG33-GFP (MOI=1). Cells were harvested 16h p.i., and GFP positive cells that co-express CD11c marker were analysed by flow cytometry (Fig 1 A). From 12h to 72h p.i., samples titration was performed on RK13 cells (Fig.1B). We observed that SG33 is able to infect ovine BM-DC (17.5 % of the cells are positive for CD11c and GFP) (Fig 1A), but the infection is abortive, unlike the infection of rabbit BM-DC (Fig 1B).

Fig.1: In vitro SG33-GFP infections



Inflammatory and humoral response after i.d. inoculations: To test the capacity of SG33 to induce immune response in sheep, i.d. immunisation with SG33-VP60 was carried out. As soon as 6 hours p.i., an in situ inflammatory infiltrate predominantly made by neutrophils was observed. Later, it appeared mostly composed of mononuclear cells, indicating the development of local progressing inflammatory response which seems proportional to the viral doses inoculated. Moreover, seroconversion to SG33 was observed after the second injection (MYXV ELISA test). An antibody response against VP60 was also detected for eight of ten inoculated sheep by RHD ELISA test [4].

Protection against BTV8 challenge: With the aim to evaluate the capacity of recombinant viruses expressing bluetongue proteins to induce protection against BTV8, we vaccinated sheep with SG33-VP2, or SG33-VP2+VP5. We observed after challenge that, in group of sheep vaccinated with SG33-VP2, only one animal presented mild clinical signs and the blood viral titres of BTV8 decreased significantly six days after challenge. Sheep vaccinated with SG33-VP2+VP5 presented more severe clinical signs and higher blood BTV8 titres, whereas sheep of the group vaccinated with the Merial vaccine presented no clinical signs and very low BTV8 blood titres. The most severe clinical signs were observed in the SG33-Ø vaccinated group (2 fatal cases).

Discussion and conclusions

The SG33 strain of MYXV is able to infect ovine BM-DC, but it turns out that these cells are not permissive for MYXV. SG33 can also induce both inflammatory and humoral responses. Theses results suggest that SG33 can be used as vaccine vector in ruminants. Finally, the fact that vaccination with SG33-VP2 can induce a significant protection against a challenge with BTV8 confirms that MYXV, and especially the SG33 strain, is a good vaccine vector for non-natural host MYXV species. Concerning the role of structural BTV proteins in the protective immune response, new studies must be conducted to try to understand the differences observed between the level of protection conferred by the two recombinant viruses used (SG33-VP2 and SG33-VP2+VP5) and the protection level with clinical score.

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ACID STABILITY PHENOTYPES IN EQUINE RHINITIS B VIRUS: SEROTYPE DESIGNATION AND RECOGNITION OF DUAL INFECTIONS

Jacquelyn HORSINGTON, James GILKERSON, Nino FICORILLI, Michael STUDDERT, Carol HARTLEY

Equine Infectious Diseases Laboratory, Faculty of Veterinary Science, University of Melbourne, Australia,

Keywords: acid-stablilty, erbovirus, serotypes

Introduction and objectives

Equine rhinitis B virus (ERBV) is the sole species in the genus *Erbovirus,* family *Picornaviridae*. Three serotypes have been identified: ERBV1, ERBV2 and ERBV3. Phylogenetic analysis based on the amino acid sequence of the capsid proteins (P1 region of the genome) assign erboviruses to three distinct phylogenetic groups that correspond with acid stability phenotype (Black *et al.,* 2005). Both acid-labile and acid-stable phenotypes of ERBV1 are recognised, while ERBV2 are acid-labile and ERBV3 are acid-stable. The genomic sequences of the acid-stable ERBV1s closely resemble that of ERBV3. Acid-labile viruses lose infectivity below pH 5. Acid stability is defined as a reduction in titre of ≤3 $\log_{10}TCID_{50}$/ml after incubation at pH 3.3 for 1 hour at room temperature. The aims of this study were;

1. To identify regions of the viral capsid that confer acid stability by isolation of acid-stable mutants of acid-labile ERBV1 after serial treatments at pH 3.3.
2. To use ERBV1, ERBV2 and ERBV3 specific antisera to examine the relationship between serotype, genotype and acid stability phenotype.

Materials and methods

The acid stability of viruses was measured in an acid stability virus titration, where virus is treated at a range of pHs (from pH 3.3 to pH 7) for 1 hour at room temperature, then returned to pH 7.0 and titrated on RK13 cells. To select for acid-stable mutants two viruses, ERBV1.1436/71 and ERBV1.2225/03, were used. Viruses were treated at pH 3.3 for 1 hour then pH was returned to 7.0 and the treated virus used to inoculate RK13 cells. The regime was repeated two times and the recovered viruses tested to confirm acid stability and the P1 region of the genome sequenced. Acid-stable viruses were plaque purified and a selection of plaques tested again by acid stability virus titration, sequenced and compared to the parent virus in serum neutralisation (SN) assays.

ERBV-specific rat antisera were produced by inoculating rats with 10 µg sucrose gradient purified virus on two occasions four weeks apart. Sera were used in western blot (WB) and SN assays against a selection of known acid-labile ERBV1, acid-stable ERBV1, ERBV2 and ERBV3 isolates.

Results

The acid-labile prototype ERBV1 strain 1436/71 was used for selection of an acid-stable mutant as described. The selected virus (1436/71AS) was shown by acid stability virus titration to be stable at pH 4 and showed only a 10-fold reduction in titre at this pH compared to the 6 $\log_{10}$ reduction seen with the ERBV1.1436/71 parent. An amino acid change in the C-terminus of the VP1 capsid protein, Tyr321→His, was the only change identified in the P1 region of the 1436/71AS virus.

Treatment of ERBV1.2225/03 at pH 3.3 resulted in the isolation of a virus (2225AS) that showed stability at this pH compared to the parent virus (1.3 $\log_{10}$ reduction in titre at pH 3.3 compared to 4 $\log_{10}$ reduction of ERBV1.2225/03 parent virus). In striking contrast to the 1436/71AS acid-stable mutant, the nucleotide sequence of the P1 region of 2225AS was an entirely distinct ERBV sequence to the parent. The sequence of 2225AS was most closely related to the prototype ERBV3 isolate, 4442/75. The detection of two different ERBV strains in a single sample suggests the horse was infected with two distinct erboviruses. This finding prompted investigation of dual infections in other isolates, and identified two other cases where both an acid-labile ERBV1 and acid-stable ERBV3 virus were present in the same sample.

In order to better understand the association of virus serotype, genotype and acid stability phenotype, monospecific rat antisera to ERBV1.1436/71, ERBV1.58-13/89 (acid-stable), ERBV2.313/75 and ERBV3.2225AS were prepared. All antisera had high SN antibody titres to the homologous virus. ERBV1.1436/71 and ERBV2.313/75 were not neutralised by heterologous antisera. Interestingly, ERBV1.58-13/89 antisera neutralised ERBV3.2225AS and ERBV3.2225AS antisera neutralised ERBV1.58-13/89.

Discussion and conclusions

A single amino acid change at residue 321 (Tyr→His) in VP1 of ERBV1.1436/71 appears to be associated with acid stability. Single amino acid changes associated with acid stability have also been reported for foot and mouth disease virus (Twomey *et al.,* 1995) and human rhinovirus (Skern *et al.,* 1991). The tyrosine at position 321 of VP1 is highly conserved among acid-labile ERBV1 whereas acid-stable ERBV3 contain a positively charged lysine, consistent with the positively charged histidine residue in ERBV1.1436/71AS.

ERBV3.2225AS is the first ERBV3 isolated in Australia, and is also the first reported occurrence of an infection with multiple ERBV serotypes in a single horse. Dual infections were subsequently found in two of ten other isolates. These results suggest dual infection may be common and may account for the previously documented cross-neutralisation of these viruses, particularly with ERBV1 and ERBV3.

Cross-neutralisation between ERBV1.58-13/89 and ERBV3.2225AS indicated the acid-stable type 1 and type 3 viruses were the same serotype. Analysis of three other available ERBV isolates considered to be acid-stable type 1 showed strong cross neutralisation antibody titres with the ERBV3.2225AS and ERBV1.58-13/89 antisera and little to no neutralisation by the ERBV1.1436/71 antisera. These results show that the viruses previously classified as acid-stable ERBV1 are in fact ERBV3. Erboviruses now clearly segregate into three distinct groups consistent with genotype, serotype, and phenotype - acid-labile ERBV1, acid-labile ERBV2 and acid-stable ERBV3.

Acknowledgements

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DEVELOPING A NOVEL EUROPEAN SURVEILLANCE SYSTEM FOR DETECTING PATHOGENS IN WILDLIFE: INTRODUCING WILDTECH

Richard G LEA[1], Lisa YON[1], Paul BARROW[1], Suzanne BOARDMAN[2], Duncan HANNANT[1]

[1]University of Nottingham, School of Veterinary Medicine & Science, Sutton Bonington Campus, Loughborough, UK;
[2]East Midland Zoological Society, Atherstone, Warwickshire, UK

Keywords: microarray, nucleic acid, pathogen, serology, technology, wildlife

Introduction and objectives
Approximately 75% of all diseases which have emerged in the last few years are of wildlife origin and a large number of these pathogens are zoonotic. Moreover, there is an increasing prevalence of zoonotic and epizootic infections in wildlife, domestic animals and humans. Despite this alarming trend, current disease surveillance of wildlife is minimal. The EU has recently funded a consortium under FP7 (WildTech) to develop and validate cutting edge molecular technologies and analytical tools to carry out surveillance of new and emerging pathogens in wildlife in European countries.

Materials and methods
Central to the work of WildTech is the generation of oligonuceotide arrays for the detection of viral, bacterial and parasitic diseases of concern across Europe. Degenerate or randomly synthesized oligonucleotides will be used to detect novel sequences in infectious agents and host response to pathogens evaluated by antibody detection in serological arrays. A novel international database is being developed for data and information input pertaining to existing and future samples and an extensive network of wildlife specialists across 22 countries will provide samples for technology development and validation. Epidemiological modelling will enable the potential risk of the emergence of new diseases to be assessed.

Results
A survey of wildlife specialists in 22 countries, in combination with reference to published lists (OIE, CDC), identified pathogens of concern in wildlife across Europe. Microarrays and serological arrays will be developed for up to 200 of these pathogens. Full validation will be carried out for up to 20 of these infectious agents. Epidemiological data and models of infection spread will be developed for these agents.

Discussion and conclusions
WildTech will produce arrays capable of screening a single sample for approximately 200 pathogens. The resulting data will greatly increase our knowledge of the epidemiology of these pathogens in wildlife populations. The consortium is working closely with the OIE and it is anticipated that these approaches will ultimately be used for monitoring disease spread in wildlife, domestic animal species and humans, both within Europe and beyond.

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EPIDEMIOLOGY AND CONTROL OF CLASSICAL SWINE FEVER IN WILD BOAR AND POTENTIAL USE OF A NEWLY DEVELOPED LIVE MARKER VACCINE

Frank KOENEN[1], Andy HAEGEMAN[1], Sándor BELÁK[2], Katinka BELÁK[3], Martin BEER[4], Matthias KRAMER[5], Volker MOENNIG[6], Vittorio GUBERTI[7], Martin HOFMANN[8], Juan Plana DURAN[9], José Manuel SÁNCHEZ-VIZCAÍNO[10], Gábor KULCSÁR[11]

[1]Veterinary and Agrochemical Research centre, Brussels, Belgium;
[2]The National Veterinary Institute (Virology), Uppsala, Sweden;
[3]The National Veterinary Institute (Pathology), Uppsala, Sweden;
[4]Friedrich-Loeffler-Institute (Virology), Island of Riems, Germany;
[5] Friedrich-Loeffler-Institute (Epidemiology), Wusterhausen, Germany;
[6]Institute of Virology, School of Veterinary Medicine Hannover, Hannover, Germany;
[7]Istituto Nazionale per la Fauna Selvatica, Ozzano Emilia, Italy;
[8]Institute of Virology and Immunoprophylaxis, Mittelhäusern, Switzerland;
[9]Fort Dodge Veterinaria SA, Vall de Bianya (Girona), Spain;
[10]Universidad Complutense de Madrid (Sanidad Animal), Madrid, Spain;
[11]Central Agricultural Office, Directorate of Veterinary Medicinal Products, Budapest, Hungary

Keywords: classical swine fever, diagnosis, epidemiology, live marker vaccine, wild boar

The present presentation summarizes the results of a FP6 funded project.

Aim 1: Development of an epidemiological for CSF eradication in wild boar

A number of epidemiological parameters where either determined by using existing published methods, data collection or by new applications for estimating them (such as transmission coefficient β and the minimum wild boar number). For the latter a user-friendly excel sheet was developed based upon the hunting bag. Subsequently, a new mathematical model based upon a metapopulation principle was designed and validated using data from previous outbreaks. This new model showed that hunting is an ineffective way to control the infection as only unrealistically intensive hunting efforts could eradicate the infection. Although in small populations (<1000 to 1500 animals) a non-intervention policy revealed to be successful, vaccination was demonstrated to be an effective tool in controlling the CSFV infection as it always reduces the epidemic peak. The chance of successful eradication of the infection is determined by the percentage of the susceptible population that is vaccinated within a short range of time. While a 60% vaccination rate of susceptible animals will lead to prompt eradication, 20% will increase the probability of endemic stability of the infection.

Aim 2: Adaptation of the C-strain vaccine baits for use in wild boar with special attention to young animals

New small spherical and cuboid baits were designed and constructed. The new 3 cm spherical bait clearly showed an improved uptake rate in young animals up to 3.5 months. However, even this new small bait was not taken up by animals younger then 3 months, probably due to the fact that they prefer suckling. This has important implications in any vaccination strategy as it has to be kept in mind that these young animals cannot be immunized in this way. Furthermore, it was demonstrated that lyophilization increased vaccine stability under field conditions and is therefore a promising method to increase bioavailability during vaccination campaigns.

Aim 3: Development of a marker vaccine and accompanying diagnostic assays and protocols

A new live marker vaccine was developed whereby the E2-region of BVDV (strain CP7) was replaced by the corresponding sequence of CSFV (*Alfort 187*). Based upon in vitro and in vivo results, it can be clearly stated that CP7_E2alf is the most suited vaccine candidate as it not only provides complete sterile immunity, independent of the application method, it is also very safe to use. In none of the animal experiments any adverse effects were noted on health or farrowing with normal birth performance and no effect on the health status of the piglets. Not withstanding the BVDV background of CP7_E2alf, no serological or virological evidence could be found for the presence of CP7_E2alf in young ruminants and rabbits upon oral application, and even intramuscular application of cattle and sheep did not result in detectable vaccine virus replication or shedding. In addition to the safety of this candidate, animals immunised with CP7_E2alf can be differentiated from wild type infected animals using either commercial ELISA system or by a real-time RT-PCR developed during this project. The robustness of the developed real-time RT-PCR was confirmed during an interlaboratory evaluation.

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THE GENOME OF CHICKEN PARVOVIRUS

László ZSÁK, J. Michael DAY

Southeast Poultry Research Laboratory, USDA-ARS, Athens, GA, USA

Keywords: chicken, enteric disease, parvovirus

Introduction and objectives

Viral enteric disease is a significant economic problem in the poultry industry throughout the world. The major enteric disease complex in broiler chickens is known as runting-stunting syndrome (RSS) (1). Although a number of different viruses, including avian reoviruses, rotaviruses, astroviruses and coronaviruses, have been isolated from the intestinal contents of birds in affected poultry flocks, currently their role in RSS is not understood. Earlier reports suggest that other viruses might also play significant role in the aetiology of RSS (2). Recently, we reported the application of a molecular screening method to detection of novel viruses in intestinal samples of chickens exhibiting characteristic signs of enteric disease (3). The technique is based on random amplification of particle-associated nucleic acids in clinical samples. Using this method, we successfully identified parvovirus DNA sequences in intestinal homogenates of affected chickens. Sequence analysis of the left end of the genome, including the complete nonstructural gene (NS), suggested that the chicken parvovirus (ChPV) represented a novel member of the Parvovirus family. The main objective of this study was to determine the full-length genome sequence of the chicken parvovirus. To improve understanding of evolutionary relationship of ChPV with previously described parvoviruses, phylogenetic analyses were performed and based on those data, classification of ChPV is proposed.

Materials and methods

The chicken parvovirus ABU strain was originally isolated from chickens in Hungary in 1984 (2). The cesium chloride purified ABU virus (kindly provided by J. Kisary) was used to inoculate SPF broiler chickens. Seven days later, the chickens were killed and the entire intestinal portion containing the chicken parvovirus ABU-P1 strain was processed for sequence analysis as previously described (2). In order to detect viruses of unknown sequence, we used PCR approach allowing sequence-independent amplification. This random PCR resulted in representative amplification of all particle associated nucleic acids (PAN) in the reaction. The specificity was not provided by the PCR but rather by selective isolation of PANs. The DNA from purified viral particles was extended using random primers containing a conserved 20-nucleotide 5' sequence and a degenerate six-nucleotide 3' end. Extended products were subsequently amplified by PCR with a primer complementary to only the conserved end of the random primer. The PCR products were cloned and sequenced, the sequences obtained were compared to those in the GenBank database using the BLASTn and BLASTx algorithms. The full-length chicken parvovirus genome was assembled using subsequent PCR assays, which were designed to close the gaps between the contigs.

Results

A total of 768 clones were sequenced. A high proportion of the clones (80%) were similar to chicken DNA, phages or viral sequences. Four contigs were assembled from 27, 24, 19, and 21 clones, respectively. These sequences had no significant nucleotide similarity in the database, but the deduced amino acid sequences from each contigs were similar to those of members of the family *Parvoviridae*. The complete chicken parvovirus genome was found to be 5,257 nt long and its organization resembled that of the other known parvoviruses. The genome was flanked by two identical inverted terminal repeats of 206 nt and contained two major ORFs encoding the NS protein and at least two capsid proteins (VP1 and VP2). Like members of the boca parvovirus genus, the chicken parvovirus also had a third, middle ORF of 306 nt. In bocaviruses, this gene encodes a nonstructural protein with unknown function. The full-length nucleotide coding sequence of ChPV showed 41.7-45.5% identity with other parvoviruses and formed a separate branch of a parvovirus phylogenetic tree (Fig. 1). The NS protein and the VP1 capsid protein had 46.0-53.5% and 42.6-57.1% amino acid similarity to prototype parvoviruses, respectively.

Discussion and conclusions

Here we present the first complete genomic sequence of the chicken parvovirus ABU-P1 strain. The *Parvoviridae* family is divided into two subfamilies on the basis of host range, the *Parvovirinae*, which infect vertebrate hosts, and the *Densovirinae*, which infect insects and other arthropods (4). Since there is no sequence homology between the two subfamilies, our phylogenetic studies included only members of the *Parvovirinae*. Our results from full-length nucleotide sequences as well as deduced amino acid sequences of the two major ORFs were consistent and confirmed that ChPV represents a new member of the *Parvovirinae*. Our data also show that this virus clearly separates from the previously described parvovirus genera and therefore we propose to classify ChPV in a new genus within the *Parvovirinae* subfamily.

Acknowledgements

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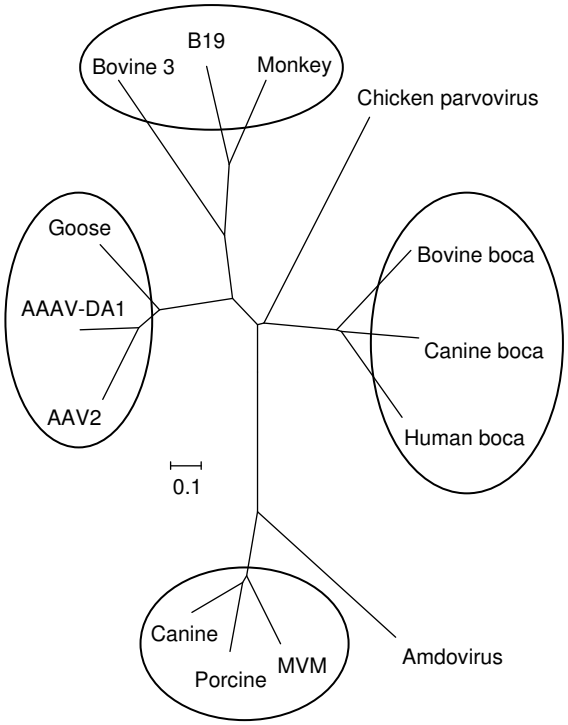

Fig.1. Evolutionary relationships between the full-length coding sequences of parvoviruses. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 5.60225337 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. There were a total of 3231 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (5).

EVIDENCE OF WITHIN-COUNTRY CLUSTERING OF CANINE PARVOVIRUSES FROM CLINICALLY-ILL ANIMALS IN MAINLAND UK

Simon R. CLEGG, Karen P. COYNE, Rosalind M. GASKELL, Susan DAWSON, Alan D. RADFORD

Small Animal Infectious Disease Group, University of Liverpool, Leahurst Campus, Chester High Road, Neston, UK, CH64 7TE

Keywords: canine parvovirus, evolution, geographical clustering, sequencing

Introduction and objectives

Evolution of viruses is a constantly occurring process. Although rare, host species jumps can lead to severe pandemics of disease. This occurred in 1978, when canine parvovirus 2 (CPV-2) emerged as a severe gastrointestinal infection of dogs, having evolved from the related feline panleukopaenia virus (FPV).

This small DNA based virus has undergone evolution since its emergence and now exists as three separate types (2a, 2b and 2c), with the original CPV-2 type dying out (2a).Type 2 is now known to only circulate in live vaccines, which can be shed by dogs post vaccination. The initial species jump of parvovirus from cats to dogs is thought to be as a result of mutations in several amino acid residues in FPV. Further key amino acid mutations are shown in the table below (adapted from (4)).

2-2a	2a- 2b	2b-2c
Met - 87 - Leu	Asn - 426 - Asp	Asp / Asn - 426 - Glu
Ile - 101- Thr	Ile - 555 - Val	
Ala - 300 - Gly		
Asp - 305 - Tyr		
Val - 555 - Ile		

The geographical distribution of CPV types 2a, 2b and 2c appears to differ widely. Most studies suggest different proportions are found in individual countries with some having mostly 2c (e.g. Italy) whereas in others (including the UK), 2a and 2b predominate (2). Recently phylogenetic analysis suggests that the newer types of CPV are not as geographically mobile between countries as at first thought (3). However, there is very little detailed information on within-country genetic variation or strain mobility.

The aim of this study was to conduct a detailed investigation of within-country genetic diversity for CPV in mainland UK, using full VP2 sequence, thus including other potential mutations in addition to those used routinely for typing the viruses.

Materials and methods

Faecal samples were obtained from clinically ill dogs presenting at PDSA Petaid hospitals across mainland UK (n=355) which required more than conservative treatment. A sample of the seven most common commercial vaccines strains were also analysed. Clinical information and signalment were supplied with the samples. DNA was extracted as described previously (1). Samples were analysed using a long range PCR for full VP2 gene. The PCR amplicons were purified and sequenced bidirectionally. VP2 sequences of suitable quality were aligned into a double stranded consensus sequence, giving a final alignment of 1755 bp (n=154). Phylogenetic analysis was carried out using Mega 4.

Results

PCR analysis of the samples revealed a CPV prevalence of 58% (206/355) within this largely unvaccinated population. Analysis of the key mutation positions within the genome of the 154 sequenced VP2 genes allowed for typing of the viruses into 2a (n=69) and 2b (n=85). No type 2 or 2c were found circulating within Britain in this study.

Phylogenetic analysis demonstrated that there was no evidence of geographic clustering at the level of CPV-type when typed using key amino acid positions, with both 2a and 2b being present throughout the country. However, when the full VP2 sequence was included, there was evidence of geographical clustering, sometimes driven by single rare amino acid mutations. For example, one cluster contained 30 sequences which were identical (Liverpool group of viruses). Twenty six of these were all from the same city, the other four were from up to 307 km away within Britain.

Discussion and conclusions

As with other studies to date, only 2a and 2b viruses were found within the UK, with neither type 2 viruses being shed from vaccinated dogs, nor 2c being found. This situation differs greatly from other countries such as Italy and supports the hypothesis that geographical clustering of CPV types appear to occur between different countries (2, 3).

Here for the first time, we also show geographical clustering of CPV strains from cities within a country. This clustering of sequence types within a locality may have important ramifications for disease control, with the potential for different 'strains' of CPV types circulating both within and between countries at any one time. The full significance of this is unknown.

As well as key amino acid mutations, such as those used in typing, another mutation was found to be circulating within Britain. Residue 137, which is responsible for driving the 'Liverpool group of viruses', is found on the beta barrel and is hidden inside the virus (5). This mutation is unlikely to alter the antigenicity of the virus due to its position, but may alter the structure of the virus. Viruses with this mutation still seem likely to contribute to severe illness in dogs and replicate within the host, due to the nature of the population samples. However, this mutation was not linked to any particular final outcome, clinical signs, breed, size of dog or age of dog.

Our data suggests that even within countries, there is evidence for geographical restriction of strains, thereby suggesting two types of transmission. In many cases, viruses are clearly transmitted locally leading to the formation of geographical clades. In other perhaps rarer cases, viruses are transmitted over larger distances. The presence of similar strains in geographically distinct regions could also indicate co-evolution, as has been suggested for the rapid emergence around the world of the initial CPV-2 in 1978.

Acknowledgements

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THE GENOME SEQUENCE OF SQUIRREL POX VIRUS

Alan D. RADFORD[1], Colin J. MCINNES[2], Alistair C. DARBY[3], Neil HALL[3], Julian CHANTREY[4]

[1]Small Animal Infectious Disease Group, Faculty of Veterinary Science, Leahurst Campus, University of Liverpool, Liverpool, UK;
[2]Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, Midlothian, UK;
[3]School of Biological Sciences, University of Liverpool, Crown Street, Liverpool, UK;
[4] Department of Veterinary Pathology, Faculty of Veterinary Science, Leahurst Campus, University of Liverpool, Liverpool, UK

Keywords: genomics, squirrel pox virus

Introduction and objectives
Squirrelpox virus (SQPV) is a poorly characterised pathogen of Eurasian red squirrels (*Sciurus vulgaris*) in the British Isles. Mortality is high in infected red squirrels and deaths caused by this virus are partly responsible for the rapid decline observed in UK red squirrel population over the last century (Sainsbury et al., 2000). Here we describe the genomic sequence of squirrelpox virus, discuss its phylogenetic relationship with other members of the *Poxviridae*, and identify novel genes potentially associated with abrogating the host immune response.

Materials and methods
The pox virus genomic DNA was cloned into SuperCos I cosmids (Stratgene, La Jolla, USA) (McInnes et al 2006). Six cosmids with an average insert size of 35 kb were selected on the basis that they spanned the entire genome. DNA was prepared (Qiagen, UK) and pooled in equimolar proportions. Sequencing was conducted on a Genome Sequencer FLX System (Roche Diagnostics, UK) and assembled using Newbler (Roche Diagnostics, UK).

Bioinformatics. After assembly, the resulting contigs were filtered with BLAST to remove the non-viral sequences. The contigs were viewed in gap4. Protein-coding genes were identified by GLIMMER, GenemarkS eukaryotic viruses model and BLASTX using a viral protein database downloaded from NCBI FTP site. Putative functions were inferred using BLAST against the National Center for Biotechnology Information databases. InterProScan Artemis v11 was used to organize data and facilitate annotation. Protein alignments for individual genes were conducted using MUSCLE.

The phylogeny was reconstructed using orthologous gene sets identified from other viral genomes using ORTHOMCL, aligned with MUSCLE and trimmed with GBLOCKS. Gene alignments were then concatenated and maximum likelihood trees calculated by JTT, estimated transition/transversion ratio, fix proportion of invariable sites using PHYML.

Structural homologues were identified using predicted amino acid sequences of relevant ORFs submitted for analysis on the SWISS-MODEL database.

Results
The sequencing and assembly resulted in 519 contigs with a total length of 259,113 bp. After the contigs were BLAST filtered to remove non-viral sequences, ten contigs of 148,445 bp remained with an average coverage of 52x. Rejected sequences were checked and found to be vector or bacterial in origin. The remaining gaps were closed by Sanger sequencing and assembly in GAP4. Details of the final assembly are shown below.

Number of bases, bp	148,803
Average sequence coverage	52x
Number of CDS	140
GC content percentage	66.69
CDS density, genes per kb	0.94
CDS average length, bp	1006
CDS coding percentage	94.7
Novel genes	29
Conserved pox/viral genes	111

Homologues were found for several mammalian genes including from the MHC, a PKR inhibitor with homology to eIF2a, 2',5'-oligoadenylate synthetase, CD47-like, and CD-48 like.

Phylogeny of 39 poxvirus conserved genes confirmed that SQPV is a distinct pox virus genus most closely related to Orf virus and Molluscum contagiosum virus.

Discussion and conclusions
SQPV is playing an important role in the demise of the red squirrel in the UK. Its genome sequence confirms the virus is quite distinct from other members of the *Poxviridae*. As with other poxviruses, the genome contains a wealth of host gene homologues that point to a role in interfering with the host immune-response to infection. These include a completely novel homologue of OAS, previously unseen in other viral species.

Acknowledgements
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PHYLOGENETIC ANALYSIS OF PRRSV FROM DANISH PIGS

Charlotte K. HJULSAGER, Solvej Ø. BREUM, Lars E. LARSEN

National Veterinary Institute, Technical University of Denmark, Copenhagen, Denmark

Keywords: phylogeny, PRRSV, sequence analysis, swine

Introduction and objectives

Porcine reproductive and respiratory syndrome virus (PRRSV) is a single-stranded RNA virus belonging to the family *Arteriviridae*. It is the causative agent of significant respiratory and reproductive disease in swine worldwide. The virus is a recently emerged pathogen, being first identified as a cause of clinical disease in 1991. The disease spread simultaneously in North America and Europe to gain global residence in a short time-span. Two genotypes of PRRSV are currently recognized due to profound genomic and antigenic differences: PRRSV EU type and PRRSV US type, named from their geographic origin of identification. Great diversity within the two genotypes exists, and further division of PRRSV EU type into at least 3 subtypes has been suggested (Stadejek et al. 2006, 2008).

In Denmark, PRRSV EU type was first identified in 1992 and a few years later the US type also was recognized. According to serologic testing, both types are prevalent in the Danish pig population. However, the genetic drift of the virus during the past 10 years has not been determined. The objective of this study was to examine the genetic diversity and evolution of PRRSV in Danish pigs by phylogenetic analysis, in order to asses the applicability of vaccines currently used to control PRRSV infection in Danish pig herds.

Materials and methods

Lung tissue from samples submitted to the National Veterinary Institute during 2003-2008 for PRRSV diagnosis were screened for PRRSV by real-time RT-PCR, essentially as described by Egli et al. 2001, on RNA extracted with RNeasy Mini Kit (QIAGEN). Complete open reading frames (ORF) ORF5 and ORF7 were PCR amplified as described (Oleksiewicz et al. 1998) and sequenced.

Sequences were aligned and Neighbour-Joining trees were constructed with ClustalX. Trees were visualized with NJ-plot software. Genbank entries of Danish PRRSV sequences from the 1990'ties were included in the phylogenetic analysis. Translated sequences were aligned with current vaccine isolates.

Results

Both PRRSV EU and US type viruses were isolated from material submitted from Danish pigs in the period 2003 to 2008. Sequences were obtained from 14 viruses isolated from different herds. There was substantial sequence diversity within both types of viruses. All Danish PRRSV type EU viruses grouped with subtype EU1 viruses. Amino acid alignments of translated sequences showed that the protein sequences were highly conserved and match the vaccine strains without differences in predicted epitope regions of ORF5 and ORF7 proteins.

Discussion and conclusions

PRRSV of both EU and US types currently are co-circulating in the Danish pig population. The viruses are diverse within both groups with a slightly higher degree of diversity within the EU type group of viruses. However, for both types, sequences match the corresponding vaccine strains. Importantly, all viruses of the EU type group with subtype EU1 viruses. Subtype EU1 contains viruses from Asia and Europe, whereas the other subtypes represent viruses from East Europe only (Stadejek et al. 2006). Introduction of new subtype viruses or drift within the present viruses could potentially affect control of PRRSV infection. Diagnostic procedures could be impaired if mutations were in primer binding sites or if they caused changes in antigenicity of viruses. Antigenic differences between EU subtypes have been demonstrated (Stadejek et al. 2008). PRRSV is a very diverse virus with a high mutation rate. It is therefore extremely important to continuously monitor and sequence the virus. The present data suggests that the PRRSV vaccines used for the moment are adequate for control of PRRSV infection in the Danish pig population.

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COMPARATIVE SEQUENCE ANALYSIS OF AVIADENOVIRUSES FROM A GOOSE AND A TURKEY

Győző L. KAJÁN[1], Andrew J. DAVISON[2], Vilmos PALYA[3], Balázs HARRACH[1], Tibor PAPP[1], Mária BENKŐ[1]

[1]Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary;
[2]MRC Virology Unit, Institute of Virology, Glasgow, UK
[3]CEVA-Phylaxia Inc., Budapest, Hungary

Keywords: adenovirus, genome analysis, goose, turkey

Introduction and objectives

There are only two members of the genus *Aviadenovirus* with fully sequenced genomes, namely fowl adenovirus 1 (FAdV-1) (1) and FAdV-9 (4). There are some further FAdVs with sequenced genome termini (2, 3). The only sequenced turkey adenovirus (TAdV) is TAdV-3 (turkey hemorrhagic enteritis virus), which is a member of the genus *Siadenovirus*. Since we are interested in the gene content and phylogeny of novel aviadenoviruses, we sequenced the complete genome of two viruses isolated in Hungary, namely a turkey adenovirus and a goose adenovirus (GoAdV). Preliminary analysis of partial hexon gene sequences showed the distinct relatedness of the GoAdV and FAdVs (5), which promised interesting conclusions regarding the evolutionary history of aviadenoviruses. Since geese are waterfowl (anseriform birds), and fowl are galliform birds, the genetic divergence of their adenoviruses might fit with a coevolutionary theory.
As the original TAdV-1 and GoAdV-1 isolates have perished, and no sequences are available from them, we propose that the isolates studied by us be named TAdV-1 and GoAdV-1.

Materials and methods

The TAdV-1 genome was sequenced by a combination of random cloning and primer-walking approaches. The virus was isolated on chicken embryo liver cells, and its DNA was purified by phenol-chloroform extraction and cleaved by *Bam*HI. The resulting fragments were ligated into the pBluescript II KS plasmid. Larger fragments were subcloned by *Pst*I enzyme. The terminal protein present at the genome ends were eliminated by alkaline treatment, and the *Bam*HI end fragments were cloned into pBluescript II KS cleaved by *Eco*RV and *Bam*HI. Primer walking and PCRs with specific primers designed from viral sequences as they were derived were also used to complete the sequence of the entire TAdV-1 genome.
The sequence of the GoAdV-1 genome was determined by shotgun sequencing. The virus was isolated on Muscovy duck embryo fibroblast cells. The DNA was extracted from the tissue culture supernatant and sheared ultrasonically. The resulting random fragments were separated by agarose gel electrophoresis and the population corresponding to 600-1500 bp was excised and extracted from the gel. The fragments were cloned using the pSTBlue1 Perfectly Blunt Cloning kit, and 750 clones were sequenced. The cloned fragments did not cover the whole genome, and the gaps were filled by PCR. The genome ends were sequenced by direct sequencing of purified viral DNA.
In both cases, the sequences were processed using the Staden program package, and the genomes were annotated with the help of the Artemis program. Predicted amino acid sequences were aligned using Tcoffee, and the alignments were edited using GeneDoc. Phylogenetic calculations were performed using the Phylip package on-line (mobyle.pasteur.fr) by the ProtDist with Categories model. The Fitch program was used by the Fitch-Margoliash method with global rearrangements for phylogenetic tree reconstruction. The trees were visualised using Mega.

Results

TAdV-1 turned out to have the longest genome (45,413 bp) and the highest G+C content (68.1%) among all adenoviruses sequenced to date The inverted terminal repeats (ITRs) of TAdV-1 are the longest among aviadenoviruses (95 bp). The GoAdV-1 genome is 43,450 bp long with a G+C content of 44.6%, and ITRs of 39 bp.
The organization of the central part of both genomes complies with that characteristic of members of the family *Adenoviridae* sequenced to date. Phylogenetic analysis and comparison of the gene content revealed a close relationship between TAdV-1 and the FAdVs. Thus, homologues are present in TAdV-1 of ORF0, ORF1, ORF1A, ORF1B, ORF2, ORF12, ORF13, ORF14 and ORF24 at the left genome end, and lipase, ORF8, ORF9, ORF11, ORF17, ORF20, ORF20A, ORF22, ORF26, ORF28 and ORF29 at the right genome end. Similar comparisons indicated a much more distant relationship between the genomes of GoAdV-1 and the FAdVs. At the left genome end, two novel ORFs and the counterparts of ORF1, ORF2, ORF12 and ORF24 are present. At the right genome end, 14 novel ORFs are present. The lipase gene homologue seems to be duplicated. Counterparts of ORF20, ORF20A and ORF22 were identified.

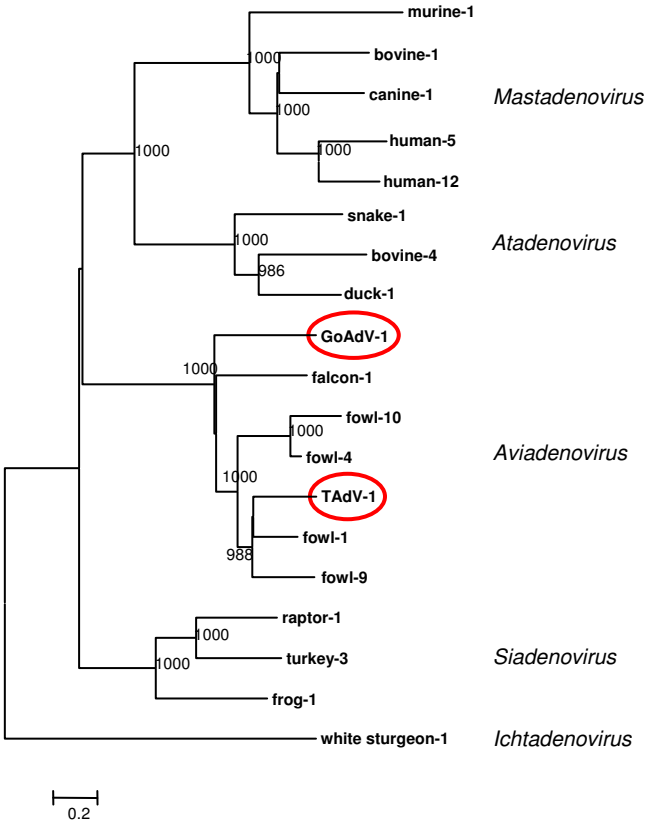

Fig. 1. *Phylogenetic tree showing a distance matrix analysis of complete hexon amino acid sequences from aviadenoviruses and some other adenoviruses. Each virus (except GoAdV-1 and TAdV-1) is labelled by its host's name. High bootstrap values indicate the reliability of the topology.*

Discussion and conclusions

The degree of phylogenetic separation between GoAdV-1, TAdV-1 and the FAdVs seems to support a coevolutionary model for adenoviruses and their hosts, in which anseriforms (waterfowl such as ducks, geese, and swans) and galliforms (fowl such as turkeys, grouse, chickens, quails and pheasants) evolved from the ancestral taxonomic group Galloanseri.
Further studies are needed to identify the function of the novel ORFs and to reveal the exact splicing patterns of the mRNAs. Nonetheless, the level of conservation within each virus genus and within the family suggests that most of the known splicing sites also exist in TAdV-1 and GoAdV-1.

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COMPLETE CSFV GENOME SEQUENCING OF RECENT CSFV ISOLATES FROM GERMANY AND COMPARISON WITH SEQUENCES OF FORMER CSFV OUTBREAKS

Immanuel LEIFER, Bernd HOFFMANN, Heinz-Günter STREBELOW, Sandra BLOME, Martin BEER

Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany

Keywords: CSFV genotype 2.3, sequencing CSFV complete genome, RT-PCR

Introduction and objectives

Classical swine fever (CSF) is a serious disease of pigs and can cause great economical losses in commercial pig productions. During the last decade, several outbreaks of CSF occurred in Europe. Most outbreaks were associated with CSFV isolates of genotype 2.3. To classify isolates, two genome fragments are routinely sequenced. One fragment encompasses 150 nucleotides of the highly conserved 5' non-translated region (NTR), the other 190 nucleotides of the genome region coding for the E2 glycoprotein (Greiser-Wilke et al., 1998). The latter is most variable. Using the sequences of the 5'-NTR, it was found that the isolates seemed to be characteristic for certain regions where outbreaks occurred, and to distinguish between isolates, they got names of the places where they were first found, e.g. 2.3*Uelzen and 2.3*Guestrow (Fritzemeier et al., 2000). Recent outbreaks among German wild boar in 2009 showed that the above mentioned nomenclature can be misleading. In order to obtain deeper knowledge about sequence differences, comparison of longer fragments seemed desirable. Unfortunately, no recent full genome sequences were available in the accessible databases. Here we report on a method specialized for sequencing complete CSFV genomes and comparing these results with sequences available in the databases.

Materials and methods

Primer sets for the amplification of the complete CSFV genome in segments of app. 1000 bases size were designed after multi-alignments of all available complete CSFV genome sequences. Segments were amplified in RT-PCR using SuperScript III Kit (Invitrogen) and subsequently sequenced using BigDye sequencing kit (AppliedBiosystems). The 5-prime and 3-prime sequences were received after 5` rapid amplification of cDNA ends (RACE) and 3` RACE RT-PCR following the manufacturers instructions (Invitrogen). Complete consensus sequences were received after alignment of all individual segment sequences using GCG software (Accelrys Inc.).

Results

Different CSFV isolates from the 2009 outbreak in German wild boar populations as well as CSFV isolates from former outbreaks in Germany were completely sequenced. The obtained sequences were used for phylogenetic analyses and compared to all related sequences available in the National Centre for Biotechnology Information (NCBI) database. The derived full length sequences were used to establish a new nomenclature for CSFV isolates.

Discussion and conclusions

CSFV isolates obtained during the 2009 outbreak among German wild boars belong to genotype 2.3. As a result, recent German CSFV isolates cluster together with most European isolates found over the last decade. Nevertheless, it was found that the historical nomenclature of CSFV isolates, which is based on the 5'-NTR pattern, can be misleading when using it for molecular epidemiology.

To get more detailed information, sequencing of longer fragments and complete CSFV genomes was carried out. To this means, a method was established that utilizes an approach where the whole genome sequence is derived from multiple segments of about 1 kb and 3'- and 5'-RACE. Furthermore, new techniques like deep sequencing with 454 technology for generation of full length sequences will be utilized.

The derived full-length sequences are used to classify recent outbreaks and facilitate molecular epidemiology. In addition, evolution of new strains can be studied on a broader base.

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PRELIMINARY REPORT ABOUT THE DEVELOPMENT OF GENOMIC AND SUBGENOMIC (SG) TAQMAN REAL-TIME RT-PCR FOR DETECTION AND QUANTIFICATION OF EQUINE ARTERITIS VIRUS

Ákos HORNYÁK[1], Tamás BAKONYI[1, 2], Norbert NOWOTNY[2], Miklós RUSVAI[3]

[1]*Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Budapest, Hungary;*
[2]*Zoonoses and Emerging Infections Group, Clinical Virology, Department of Pathobiology, University of Veterinary Medicine, Vienna, Austria;*
[3]*Department of Pathology and Forensic Veterinary Medicine, Faculty of Veterinary Science, Szent István University, Budapest, Hungary*

Keywords: abortion, EVA, Nidovirales, real-time RT-PCR, transcription strategy

Introduction

Equine viral arteritis (EVA) is an acute contagious viral disease of *Equidae* present worldwide, and characterized by fever, catarrh, oedema, and, most importantly, abortion (5). However, the asymptomatic form of the infection is the most frequent. As other members of the *Nidovirales*, EAV has a unique transcription strategy for all structural protein encoding genes; producing 3' co-terminal nested set of subgenomic mRNAs (sg-mRNAs) (2). In our work we exploited the presence of the sg-mRNAs in cells where intensive virus replication are taking place. Genomic (1) and subgenomic Taqman real-time RT-PCRs developed by us are capable to distinguish between replicating and inactive viruses enabling to establish comprehensive diagnosis of the EVA infection.

Materials and methods

Virus of known titre, propagated on RK-13 cells, supernatants stored at -80 °C and two semen samples; one from a persistently infected carrier stallion and an EAV negative one were applied for assessment of the primers and probe check. RK-13 cells in plastic tissue culture plate with a diameter of 2 cm^2 each well were infected with Bucyrus strain of 10E3 and 10E1 concentration. Following one hour adsorption period inocula were replaced with EMEM containing 2% NCS. At each 8 hours supernatants and infected cells were collected separately up to the 48 hour p. i. and stored at -80 °C. Alignment was constructed with sequence data of 35 and 170 EAV strains on the leader region and on the N gene respectively. Primers and probe were designed on the leader sequence and the N gene of the Bucyrus virus, using Primer Designer 4 for Windows 95. Downstream primers annealing sites are at nucleotide (nt) position 185 (leader region) and 12248 (membrane protein encoding gene), upstream primer is located at nt position 12398 (N gene). Probe anneals at nt position 12350 (N gene) and was labelled with 6' FAM and BHQ. Taqman real-time RT-PCR was carried out by Platinum® *Taq* DNA Polymerase kit (Invitrogen), in Applied Biosystems 7300 thermocycler with the following thermoprofile; 48 °C 15 min. 95 °C 2 min. 95 °C 30 sec. 60 °C 1 min. The latter two steps were repeated 45 times, data collection were set during each last steps.

Results

Both real-time systems could detect fluorescence signals from the EAV containing samples; however the sensitivity of the genomic system was much better. No aspecific positive signals could be measured with selected other pathogens causing abortion in mares; EHV-1, EHV-4, *Chlamidophyla psittaci, Leptospira icterohaemorrhagiae, Salmonella abortusequi*. The mean threshold cycle values (Ct) were as follows; Subgenomic real-time RT-PCR 28.55, 31.70, 35.70, 41.73 and 43.95 with the virus assigned 540/64 with an infective titre 10E5.5, 10E4.5, 10E3.5, 10E2.5, 10E1.5 respectively. The genomic real-time RT-PCR had mean Ct values with the same sequential virus concentrations; 21.61, 24.87, 28.27, 31.29, 36.08. The EAV positive semen samples' RNA examined, produced positive fluorescence signal, mean Ct values were 30.77 and 24.26 in the subgenomic and genomic system respectively. The negative semen sample proved to be negative checked by our systems. Ct values of supernatant and cell culture derived sg-mRNA were on the same level at each time point, from the 16 h p.i. up to the 48 h p.i. in a range of 43.19 to 19.72 on a negative exponential slope.

Discussion

There is a great necessity to determine the viability and the replication ability of the viruses in the different organs and cell cultures. All the members of the *Nidovirales* serve an excellent solution for this problem due to their unique replication's strategy downstream from the replicase gene. So the structural proteins transcription produces a nested set of sg-mRNAs, which are at the same time indicators of a more or less intensive virus replication event (4). Our real-time sg-mRNA and genomic RNA detection systems are based on this phenomenon of the EAV transcription. Examining supernatants the genomic PCR outranged in sensitivity the subgenomic one, which was experienced earlier by other authors with SARS virus as well (3). In turn, cell cultures contained much more amount of sg-mRNAs, here the ration of genomic and sg-mRNAs was 1:1. Further large scale examinations are needed for the perfect optimization of both two PCR systems, which would be an excellent molecular biological tool for the EAV diagnosis either from direct samples, or following inoculation from the most sensitive cell cultures.

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PESTIVIRUS PHYLOGENY: METHODS, STRATEGIES AND DISCREPANCIES

Lihong LIU[1], Hongyan XIA[1, 2], Claudia BAULE[1], Sándor BELÁK[1, 2], Niklas WAHLBERG[3]

[1]*Joint R&D Division in Virology, The National Veterinary Institute & The Swedish University of Agricultural Sciences, SE-751 89 Uppsala, Sweden*
[2]*Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, SE-751 89 Uppsala, Sweden*
[3]*Laboratory of Genetics, Department of Biology, University of Turku, 20014 Turku, Finland*

Keywords: Bayesian, classification, maximum likelihood, pestivirus, phylogeny

Introduction and objectives
The genus *Pestivirus* of the family *Flaviviridae* consists of four approved species: *Bovine viral diarrhoea virus 1* (BVDV-1), *Bovine viral diarrhoea virus 2* (BVDV-2), *Border disease virus* (BDV) and *Classical swine fever virus* (CSFV); and a fifth tentative species, Pestivirus of giraffe (1). As more and more pestiviruses have been newly detected, it is highly needed to reveal their phylogenetic relationship with the recognised species. The aim of this study was to compare the tree topologies inferred from different methods, namely Neighbour-Joining (NJ), Maximum likelihood (ML) and Bayesian approach under two strategies: single gene analysis and analysis of a combined dataset.

Materials and methods
A total of 52 pestiviruses were used in this study, including reference strains of the four recognised species and most representative strains within species. The 5´UTR and the complete N^{pro} and E2 gene sequences were retrieved from GenBank, and combined in the same order using the software WinClada (2).

Results
Single gene analyses of pestiviruses resulted in nine different tree topologies at the species level, which in turn depicted nine different relationships among pestiviruses. By contrast, the strategy of analysing the combined sequence dataset by ML and Bayesian methods resulted in a single, well-supported tree topology, indicating a reliable and accurate pestivirus phylogeny.

A closer relationship between BVDV-3 and the recognised BVDV-1 and BVDV-2 was strongly supported by a posterior probability value of 0.99 and a bootstrap value of 69%. Relative to the divergent antelope virus, the clade of the Pestivirus of giraffe was placed in the same side of the tree as BVDV, indicating that these pestiviruses of mainly bovine-origin share the most recent common ancestor. The results are in good agreement with previous report (4). NJ analysis of the combined dataset was unable to reveal such a relationship.

Discussion and conclusions
The comparative analyses indicate that different strategies and methodologies may result in considerable discrepancies in the tree topologies during the phylogenetic analysis of pestiviruses. Thus, the careful selection of strategies and methodologies is an important issue in the phylogenetic analysis of RNA viruses.

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HEPATITIS E VIRUS IS PREVALENT IN THE DANISH PIG POPULATION

Solvej Ø. BREUM[1], Charlotte K. HJULSAGER[1], Nilsa DE DEUS[2], Joaquim SEGALÉS[2], Heléne NORDER[3], Blenda BÖTTIGER[4], Lars E. LARSEN[1]

[1]Department of Veterinary Diagnostics and Research, National Veterinary Institute, Technical University of Denmark, Copenhagen V, Denmark; [2]Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain; [3]Department of Virology, Swedish Institute for Infectious Disease Control, Solna, Sweden; [4]Department of Virology, Statens Serum Institut, Copenhagen S, Denmark

Keywords: Danish pig, HEV, zoonosis

Introduction and objectives

Hepatitis E virus (HEV) is responsible for major outbreaks of acute hepatitis in humans from developing countries, but evidence increases that also in industrialized countries locally acquired HEV infections occur. The symptoms of the disease deteriorate by increasing age, with a general mortality of 0.2-4%. A higher mortality is seen for pregnant women of 10-20% which may lead to death of either mother and foetus or the baby shortly after birth.

HEV sequences worldwide can be classified into four major genotypes. Genotypes 1 and 2 are causing the majority of HEV infections in humans in endemic areas such as Asia, Africa and Mexico. HEV of genotype 3 has been identified worldwide with an increasing frequency in human sporadic cases from regions like Europe, USA, Argentina, Japan and Australia. Furthermore, the HEV genotype 3 has been isolated from domestic pigs of the same countries. HEV genotype 4 is found mainly in Asian countries where it has been isolated from both humans and domestic pigs.

Direct evidence of zoonotic HEV transmission has recently been reported in Japan where cases of acute hepatitis E were linked epidemiologically and genetically to the consumption of uncooked pig livers and wild deer meat. Furthermore, HEV has been detected in pig livers sold in grocery stores in countries like the Netherlands, United States and Japan and HEV contaminated pig livers have been shown to contain infectious virus.

The aim of this study was to clarify if HEV is prevalent in the Danish pig population and to examine the phylogenetic relationship of HEV isolates from Danish pigs with human isolates. The presence of HEV was examined by real time RT-PCR detection of HEV RNA and serological screening for HEV antibodies. Selected HEV positive samples from Danish pigs were sequenced.

Materials and methods

Faeces and serum samples were collected from Danish pigs. RNA was purified from the samples by QIAamp Viral RNA Mini Kit (Qiagen, Germany). A real time PCR assay against a conserved region in HEV *orf*2 gene was designed using Beacon Designer 4 (Premier Biosoft Int., USA). The assay was based on the primer-probe energy transfer system (PriProET). Serum samples from Danish sows were tested for specific anti-HEV IgG antibodies by ELISA technique as previously described (5). HEV RNA from 8 positive pig samples were sequenced as described in Norder et al., 2009 (4).

Results

A real time RT-PCR assay for the detection of HEV was designed. An alignment of sequences from all four HEV genotypes from both humans and swine showed that the HEV assay can detect all four genotypes. Since the HEV assay is based on the PriProET technique a melting point analysis of the probe target sequence will provide an indication of the HEV genotype. The performance of the HEV real time RT-PCR assay was tested on a plasmid containing the HEV *orf*2 gene and showed a high PCR efficiency of 95% and broad dynamic range of 7 Log. To exclude false negative PCR results 21 randomly selected HEV PCR negative faecal samples were tested for PCR inhibition by addition of positive HEV material. No PCR inhibition was found for any of the tested samples.

HEV RNA was detected in 49.5% (48 out of 97) faecal samples from Danish pigs aged 1 to 5 months (Table 1). The frequency of HEV positive faeces was significantly lower (p = 5.3E-05) in the group of pigs aged 1 to 2 months than in the group of pigs aged 2 to 5 months. Twenty-two out of the 40 tested herds (55%) yielded at least on pig positive for HEV RNA. Based on the melting point analysis of the real time RT-PCR assay all HEV positive samples belonged to HEV genotype 3 or 4.

Serum samples from 213 Danish sows of 71 herds were analysed for the presence of HEV IgG antibodies. In total, 91.6% (65 out of 71) of the herds were seropositive for at least one sow, corresponding to 73.2% (156 out of 213) of the examined animals. From 22 of the HEV seropositive herds with at least 2 positive sows, a total of 44 serum samples from piglets aged 8 to 12 weeks were analysed for the presence of HEV RNA and 10 (22.7%) of them, coming from 7 herds, were positive.

Sequence analysis of 8 HEV isolates from Danish pigs showed that they all belong to genotype 3. Phylogenetic analysis showed high similarity between HEV isolates from Danish pigs and HEV of genotype 3 from Danish patients.

Table 1. Detection of HEV RNA in faeces of Danish pigs

Age (weeks)	No. of pigs tested	No. of HEV positive pigs (%)	No. of herds tested[a]	No. of HEV positive herds (%)
4 – 8	32	7 (21.9)	19	5 (26.3)
9 – 12	33	18 (54.5)	16	9 (56.3)
13 – 22	32	23 (71.9)	14	11 (78.6)
Total	97	48 (49.5)	40	22 (55.0)

Discussion and conclusions

The present study shows that HEV is highly prevalent in Danish pig herds. The obtained HEV prevalence of 49.5% in faeces of Danish pigs aged from 4 to 22 weeks was higher than that found in a Spanish study (17.4%) including similar age groups (3). Other studies from USA, UK and Italy found HEV prevalences of 22 to 42% in pigs aged 8 to 17 weeks, which is more similar to the high HEV prevalences of 54.5% (9 - 12 weeks) and 71.9% (13 - 22 weeks) we found in similar age groups (1, 2). The higher prevalence of HEV in faeces of Danish pig could be explained by several factors, such as the real time PCR assay used is more sensitive than the nested PCR assays used in previous studies, PCR inhibition was not present or a change in the epidemiology of HEV infection has occurred.

The HEV seroprevalence of 73.2% found in Danish sows fits well with studies from Sweden, Spain and United Kingdom in which seroprevalences between 58% and 85% were found (1, 5). This indicates that HEV circulate in most pig herds and that most pigs become infected with HEV during their growth period.

The PriProET real time PCR assay results indicated that all HEV positive pig samples belonged to genotype 3 or 4. This was confirmed by sequence analysis of some HEV positive pig samples showing that they belonged to genotype 3. Phylogenetic analysis of the HEV isolates from Danish pigs showed high similarity with HEV genotype 3 strains from Danish patients (4). Similar high homologies between HEV strains of genotype 3 from pigs and humans have also been found in other studies.

In conclusion, our results show that HEV is highly prevalent in the Danish pig population with a HEV seroprevalence of 92% in "sows" herds and HE virus excretion from half of the analysed pig herds. The importance of HEV in Danish pigs as a cause of diseases is unknown, but the abundant presence of pigs excreting HEV raises concerns of potential zoonotic transmission of the virus.

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CIRCULATION OF A BVD VIRUS TYPE 2 CLOSELY RELATED TO THE NORTH AMERICAN HYPERVIRULENT VIRUSES IN BELGIUM

Carine LETELLIER[1], Bart PARDON[2], Piet DEPREZ[2]

[1]*Veterinary and Agrochemical Research Centre, Virology, Brussels, Belgium;*
[2]*Faculty of Veterinary Medicine, Department of Large Animal Internal Medicine, Ghent University, Ghent, Belgium*

Keywords: Bovine viral diarrhoea virus (BVDV) type 2, high virulent virus

Introduction and objectives
Since the wide spread outbreaks of severe acute bovine viral diarrhoea virus (BVDV) type 2 during the years 1993-1995 in North America (Ridpath et al., 2006), investigations have been conducted to assess the BVDV diversity, in particular the presence of such virulent type 2 strains in Europe. Even if BVDV-2 was identified in many European countries such as Belgium, France, UK, Italy, Slovakia, Austria,.. these viruses have not been associated to severe epidemics. BVDV-2 viruses were first identified in Belgium in 1991 and are periodically isolated since that time. The aim of this study was to investigate a BVDV-2 hemorrhagic case and to characterize the virus.

Materials and methods
Case 1 (07/3913): in April 2007, a cow presenting uterine and intestinal hemorrhages was brought to the veterinary clinic and died. Case 2 (09/50AS): In February 2009, a Belgian blue bull calf of 1,5 years of age presenting acute colic and decubitus was brought to the veterinary clinic and died also. The 2 cases were examined for the presence of BVDV by real time RT-PCR (Letellier and Kerkhofs, 2003). Virus isolation was performed on MDBK cells. PCR amplification and sequencing of part of the 5'UTR and Npro regions were performed. Sequences from 07/3913 and 09/50AS isolates were aligned with other BVDV 2 sequences, from Belgium (covering the 1991-2008 period) or retrieved from databases. Multiple sequence alignments were generated with the program CLUSTAL of the WEMBOSS package and visualized using the Genedoc package. Evolutionary distances between sequences were estimated using Kimura-2 parameter method. Phylogenetic analyses were conducted by using the Neighbor-Joining algorithm of the *MEGA* version 4 software.

Results
Post mortem examination of case 1 revealed edema and hemorrhages of the uterus wall. Case 2 presented bloody diarrhea, straining and few erosions in the mouth. Real time RT-PCR was directly performed on organ (uterus) of case 1 and blood of case 2. Both samples were positive for BVDV 2. BVD virus was isolated on cell culture from both cases. However, virus 07/3913 was non cytopathogenic while cytopathogenic virus was isolated from blood 09/50AS, showing that the calf died from Mucosal Disease. PCR amplification and sequencing of part of the 5'UTR and Npro regions were performed. Pairwise sequence alignment showed 100% identity between virus 07/3913 and virus 09/50AS, both in the 5'UTR and the Npro regions. Phylogenetic analysis based on the alignment of the 5'UTR was shown because more sequences were available in databases. This analysis confirmed that both isolates were classified as BVDV-2 and clustered with North American isolates (fig.1). The same results were obtained for Npro (data not shown). Furthermore, the maximum identity in the Npro region was recorded between the 07/3913 and a North American sequence (P11Q).

Discussion and conclusions
The results of the phylogenetic analysis targeting both the 5'UTR and the Npro were similar and showed that the BVDV-2 viruses isolated in Belgium were classified within the BVDV-2a subgroup. However, while the majority of the BVDV 2 viruses were grouped together, suggesting circulation of local strains, the 07/3913 and 09/50AS viruses clustered with the North American BVDV-2 virus, and in particular with viruses responsible for hemorrhagic syndrome and severe outbreaks. Two other sequences clustered also with the North American isolates but no additional information is available (Vilcek et al. 2001, 2003). The origin of the viruses characterized here is not known. Indeed, livestock is usually imported from continental Europe (Germany, France,...). Severe outbreaks linked to the use of IBR vaccine contaminated with a BVDV-2 strain have been encountered in the Netherlands and in Italy but the virus isolated here is genetically distinct from the WG4622 virus isolated from a contaminated batch of the vaccine (Couvreur et al; 2002). However, there is regular importation of semen from North America. The 2 affected herds are located close to each other, but without contact between the animals or pastures. They did not purchase animals, except bulls in herd 1 but these animals were tested BVDV negative. Herd 1 was screened in 2007 for the presence of PI animals and herd 2 is currently being screened. No PI was found so far. Afterwards, animals were vaccinated. Even if no severe epidemic was recorded, a virus closely related to strains isolated during severe outbreaks in North America circulates in Belgium since 2007. This is the first isolation and characterization of such virus in Europe. This finding highlights the risk of introduction and establishment of novel hypervirulent viruses, in particular via artificial insemination.

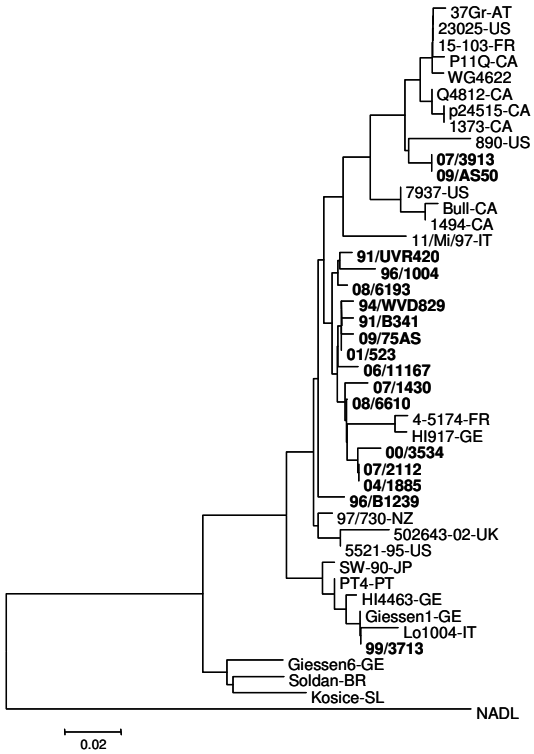

Fig.1. Genetic typing in the 5'UTR region of BVDV 2 isolates 07/3913 and 09/50AS. The Belgian isolates are shown in bold. Access numbers from database are: 37Gr: EU327594, 2302: AF039172, 15-103: AF298055, p11Q: AY149215, Q4812: L32892, p24515: AY149216, 1373: AF145967, 890: U18059, 7937: AF03975, Bull: L32894, 1494: L32893, 11/Mi/97: AJ293603, 4-5174: AF298063, HI917: AY379545, 97/730: AF026770, 502643: AY161304, SW-90: AB003622, PT4: AY944291, HI4463: AY379546, Lo1004: AM49823, Giessen6: AY379547, Soldan: U94914, Kosice: EU360934.

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ANTIVIRAL ACTIVITY OF PLANT EXTRACTS AGAINST ANIMAL VIRUSES

Viola GALLIGIONI, Francesca VACCARI, Laura GALLINA, Alessandra SCAGLIARINI

Faculty of Veterinary Medicine, Department of Veterinary Public Health and Animal Pathology, University of Bologna, Italy

Keywords: antiviral, MAPs

Introduction and objectives

Antivirals have only few applications in veterinary medicine, despite their positive impact in reducing the socio-economic consequences of disease outbreaks and in decreasing pain for pets, meeting the emotional needs of owners too.

Medicinal and Aromatic Plants (MAPs) used in traditional veterinary practices have been recorded all over the world, especially in developing countries (1), but there are few scientific studies on the evidence-based veterinary phytoterapeuticals with repeatable data and R&D on plant-based products is still lacking.

In this study *in vitro* antiviral activity of plant extracts was evaluated against RNA and DNA animal viruses.

Materials and methods

Plant extracts from *Aesculus hippocastanum, Cinnamosma macrocarpa, Cinnamosma madagascariensis, Combretum micrantum, Harungana madagascariensis, Hippophae ramnoides*, *Myrtus communis* and *Vaccinium mirtillus* were tested on growing cells in order to evaluate cell toxicity (CC50) using the MTT assay (2). The extracellular and intracellular antiviral activities (IC50) were evaluated by cytopathic effect (CPE) inhibition assay in confluent cells monolayers against RNA viruses, like Canine Distemper Virus (CDV) and Betanodavirus, and DNA viruses, like Bovine Herpes Virus type 1 (BHV-1) and Orf virus.

To ascertain test performances synthetic compounds with well known antiviral activity (i.e. Ribavirin (3) and HPMPC) were used as positive controls.

Therefore the selectivity index (SI), obtained by the ratio of the cytotoxicity and the antiviral activity values, was calculated.

Results

All the plant extracts showed only intracellular antiviral activity.

Against CDV, negative ssRNA, *Hippophae ramnoides*, *Myrtus communis* ed *Aesculus hippocastanum* were active with a SI greater than Ribavirin.

Against Betanodavirus, positive ssRNA responsible for the encephalopathy and retinopathy (VER), *Myrtus communis, Harungana madagascariensis, Combretum micrantum, Cinnamosma macrocarpa* and its fractions and *Cinnamosma madagascariensis* were active.

Vaccinium mirtillus was active against Orf virus and *Aesculus hippocastanum* was active against BHV-1, even if the SI was lower than HPMPC.

Discussion and conclusions

Plant extracts are widely used for their biological activities, but their efficacy against animal viruses have not been studied in depth yet. These preliminary results show that MAPs could be an important source for new antiviral molecules, with the possibility of development of innovative therapies.

Further studies will add information on the specific target of the compound and its possible application in clinics.

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Posters

CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS (CCHFV) AND WEST NILE VIRUS (WNV): MOLECULAR DIAGNOSIS AND TICKS SURVEY IN TURKEY

Harun ALBAYRAK[1], Emre OZAN[1], Mitat KURT[2]

[1]*Veterinary Control and Research Institute, Virology Laboratory, Samsun, Turkey;*
[2]*Veterinary Control and Research Institute, Parasitology Laboratory, Samsun, Turkey*

Keywords: CCHFV, Nested RT-PCR, rRT-PCR, tick, turkey, WNV

Introduction and objectives

Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne virus of the genus *Nairovirus* in the family *Bunyaviridae*. Crimean-Congo hemorrhagic fever (CCHF) virus is known to be transmitted by *Hyalomma* ticks. Although *Hyalomma* ticks are one of the major vectors of CCHFV, at least 31 tick species harbor the virus. West Nile virus (WNV) is a member of the Flavivirus genus, within the family Flaviviridae, belonging to the Japanese encephalitis group, such as Japanese encephalitis virus (JEV), Usutu virus (USUV), Saint Louis encephalitis virus (SLEV), and Murray Valley Fever virus (MVFV). Transmitted by mosquito bites, WNV affects a wide range of vertebrates, including mammals, birds, reptilians, and amphibians. The aim of this study is to determine presence of WNV and CCHFV and to evaluate the tick fauna of the region of endemicity, to establish what tick species harbor the viruses in Turkey

Materials and methods

A total of 1790 adult ticks were collected from cattle (302 pools, 782 ticks in total), sheep (81 pools, 636 ticks in total), goat (36 pools, 357 ticks in total), buffalo (1 pool, 1 tick in total) and turtle (1 pool, 14 ticks in total) in coastal and inland of Blacksea Region (Samsun, Sinop, Ordu, Giresun, Tokat, Amasya, Sivas) in Turkey. According to tick size, the pools range from 1 to 20 ticks. 11 different tick species (*Hyalomma marginatum marginatum* ticks (106 pools, 279 ticks in total), *Hyalomma anatolicum excavatum* ticks (32 pools, 71 ticks in total), *Hyalomma anatolicum anatolicum* ticks (2 pools, 10 ticks in total), *Hyalomma detritum* ticks (28 pools, 58 ticks in total), *Hyalomma aegyptium* ticks (1 pools, 14 ticks in total), *Rhipicephalus bursa* ticks (86 pools, 501 ticks in total), *Rhipicephalus turanicus* ticks (103 pools, 641 ticks in total), *Ixodes ricinus* ticks (58 pools, 207 ticks in total), *Haemaphysalis punctata* ticks (1 pools, 1 ticks in total), *Haemaphysalis sulcata* ticks (1 pools, 1 ticks in total), *Dermacentor marginatus* ticks (3 pools, 7 ticks in total)) were recognized on the animals in the region. Tick pools were surveyed for the presence of West Nile virus (WNV) and Crimean-Congo hemorrhagic fever virus (CCHFV) by Taqman-based real-time reverse tarnscriptase polymerase chain reaction assay (rRT-PCR) and nested RT-PCR, respectively.

Results

Although no WNV nucleic acid was detected from tick samples, CCHFV nucleic acid was found in 6.88% (29/421) of the tick pools. According to this result; positivity rate of provinces were detected 4.83% (3/62), 4.34% (1/23), 6.32% (5/79), 14.63% (12/82), 5.55% (3/54), 3.38% (2/59), 4.83% (3/62) for Samsun, Ordu, Giresun, Sinop, Amasya, Tokat and Sivas respectively. CCHFV nucleic acid was detected from 7 tick species. Except buffalo and turtle, viral nucleic acid was detected from ticks found on cattle, sheep and goat.

Discussion and conclusions

Eleven tick species were recognized on the animals in the surveyed region. The most abundant species were *Rhipicephalus turanicus*, *Rhipicephalus bursa*, *Hyalomma marginatum marginatum* and *Ixodes ricinus* with 35.81% (641/1790), 27.98% (501/1790), 15.58% (279/1790) and 11.56% (207/1790), respectively. *Hyalomma detritum* ticks represented 3.24% (58/1790) of the total number of ticks. *Hemaphysalis sulcata*, *Hemaphysalis punctata, Dermacentor marginatus* and *Hyalomma aegyptium* were the minor species and represented 0.05% (1/1790), 0.05% (1/1790), 0.39 (7/1790) and 0.78% (14/1790) of the tick population, respectively. I.ricinus was found in narrow five localities (Korgan, Ordu, Bulancak, Giresun and Resadiye).

In conclusion, the results of the present study demonstrate the presence of CCHFV in 7 tick species. Even though *Hyalomma* is the main vector for CCHFV, but other 7 tick species may play a role in CCHFV transmission.

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SEROEPIDEMIOLOGY OF THE ARBOVIRUS OF CRIMEAN-CONGO HEMORRHAGIC FEVER IN RURAL COMMUNITY OF BASRA

Adel ALYABIS, Hassan J. HASONY

Laboratory Department ,Microbiology and Serology Subdepartment,General Hospital,Basra, Iraq

Keywords: seroepidemiology

Introduction and objectives
In 1979 cases of Crimean-Congo Hemorrhagic Fever (CCHF) were recognized in Iraq, following years several cases of CCHF were diagnosed in Basra, southern Iraq. A seroepidemiological survey was carried out in rural community of Basra to estimate the size of the enzootic focus in which the CCHF virus is circulating. A total of 682 serum samples were collected from apparently healthy individuals, their ages range from 5 to 76 years, 20% of the collected sera were obtained from occupational risk groups (veterinarians, abattoirs workers, farmers). Sera collected from 74 sheep and 48 cattle, 42 tick pools were also gathered parallel to the human and animals sera in the same areas.

Materials and methods
Enzyme-linked immunosorbent assay (Elisa) was used to detect the prevalence of circulating IgG antibodies in the collected sera.

Results
In general IgG antibodies against CCHFV were found in 4.3% of the resident in rural areas of Basra. Seropositive sera were detected in 9.7% of northern residents, while 20% of the sheep sera and 37% of cattle sera were seropositive which indicate that the virus is circulating in the area within the endemic level. The tick pools were identified, the predominant tick species was diagnosed as *Hyaloma marginatum*.

Discussion and conclusions
The existence of enzootic focus for the CCHFV is maintained by ecological, socioeconomic variables, the possibility of emerging infections should always be considered.

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SEROSURVEY OF ITALIAN DOGS FOR EXPOSURE TO INFLUENZA A VIRUSES

Paola DE BENEDICTIS[1], Tara C. ANDERSON[2], Elisabetta VIALE[1], Crispina VEGGIATO[1], Silvia TIOZZO CAENAZZO[1], P. Cynda CRAWFORD[2], Ilaria CAPUA[1]

[1]OIE/FAO and National Reference Laboratory for Newcastle Disease and Avian Influenza, OIE Collaborating Centre for Epidemiology, Training and Control of Emerging Avian Diseases, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padova, Italy;
[2]College of Veterinary Medicine, University of Florida, Gainesville, Florida, United States of America

Keywords: canine, H3N8, influenza A virus, Italy, serosurvey

In 2004, the direct interspecies transmission of an H3N8 equine influenza A virus to dogs was reported in the United States. The transmission of an H3N2 avian influenza A virus to dogs was detected in South Korea in 2007. Both viruses have adapted to dogs, cause influenza-like illness, and are sustained in the canine populations by horizontal transmission. Interspecies transmissions of H3N8 equine and H5N1 avian influenza viruses have also been reported in dogs in the United Kingdom, Australia, and Thailand; however no evidence of sustained horizontal transmission has been found. These reports have increased awareness regarding the potential role of dogs in influenza A virus ecology. Multiple influenza A virus outbreaks have occurred in poultry (H7N1, H7N3) and horses (H3N8) in Italy since 2000. The objective of this study was to determine the prevalence of dogs in Italy that have been exposed to influenza A viruses. We conducted a retrospective survey of 6,859 canine serum samples, collected throughout Italy from 2006 to 2008, for antibodies to influenza A nucleoprotein and several hemagglutinin proteins using multiple testing formats (competitive ELISA, hemagglutination inhibition and immunofluorescent antibody assays). The overall prevalence of influenza A virus exposure in dogs was 0.03% (2/6,859). Seropositive dogs had antibodies to influenza A nucleoprotein and the H3 hemagglutinin proteins from recent H3N8 canine and equine viruses. This low prevalence of exposure suggests that dogs do not currently play a significant role in influenza A virus ecology in Italy.

INACTIVATION OF FELINE CALICIVIRUS AND BACTERIOPHAGES AS MODELS FOR HEPATITIS E VIRUS IN FOOD MATRICES

Eva EMMOTH, Frederik WIDÉN, Irene DERGEL, Sándor BELÁK

Department of Virology, Immunobiology and Parasitology, National Veterinary Institute (SVA), SE-751 89 Uppsala, Sweden

Keywords: food, hepatitis E, inactivation methods

Introduction and objectives

HEV genotype 3 in swine is considered zoonotic and has been demonstrated to infect humans through a food borne route (1). Food considered as a risk is primarily swine liver but also pork meat, dry ham and fermented sausages.

The objective of this task was to test methods for inactivating hepatitis E (HEV) virus in food matrices for which the model system of feline calicivirus (FCV), a known model (2) was used, as HEV does not replicate in cell culture. As an alternative model system, bacteriophages were considered, both structurally and genetically similar and other types. The advantage of the bacteriophage system is that propagation easily is performed in bacteria and quantification techniques are cheap and simple to perform.

Various inactivation methods were assessed for their effect on viruses present in the food matrices: high pressure, lactic acid and intense light pulses.

Materials and methods

Viruses used were feline calicivirus, strain FCV-2280 and FCV wild-type (SVA). Bacteriophages used were Enterobacteria phage MS2, Enterobacteria phage ØX174, and Salmonella typhimurium phage 28B.

Pieces of raw swine liver, pork meat, dry ham and fermented swine sausage were spiked with virus 1/10, and subjected to various inactivation methods at ambient temperature: A high pressure device mediated by water applying pressures of 200-600 Mpa for 1-10 min; lactic acid in concentrations 0.5 -2.2 M for 10 min; Intense light pulses applied at a distance of 14 cm and 1-20 pulses at 3000 V per cm^2.

Results

High pressure: For swine liver, a pressure of 400 MPa for 1 min inactivated 4 log_{10} of FCV-2280 and 1-5 min at 300 MPa was needed to inactivated 4.1 log_{10} of FCV-SVA. No significant inactivation of the bacteriophages could be seen. For dry ham, 1.5 log_{10} reduction of FCV-2280 is achieved after 10 min at 500 MPa, with a final inactivation of 3.9 log_{10} after 10 min at 600 MPa. For FCV-SVA, 3.7 log_{10} inactivation is seen after 10 min at 500 MPa, with a final inactivation of 3.9 log_{10} after 10 min at 600 MPa. After 10 min at 600 MPa, MS2 was inactivated by 1.3 log_{10}, ϕX174 by 2 log_{10}, and phage 28b by 1.7 log_{10}.

Lactic acid: For swine liver, a significant reduction of about 2 log_{10} for both FCV was seen at 2.2 M lactic acid, pH for 10 min.For pork meat and both FCV, reductions of 3 log_{10} was achieved after 10 min at 2.2 M lactic acid pH 3. At this concentration and pH, the MS2 bacteriophage showed a small reduction of 1 log_{10}, while the bacteriophage ØX174 was even more insensitive.

Intense light pulses: Results will be presented.

Discussion and conclusions

Regarding **high pressure**, FCV in dry ham was more resistant than in liver, dry ham has a higher salt content. 5-600 MPa is needed to achieve a good inactivation. The bacteriophages show 1-2 log_{10} inactivation at the highest pressure and time used. For swine liver, lower pressures are needed for FCV, liver has a higher water content.

Regarding **lactic acid,** a higher inactivation of FCV by 1 log_{10} is seen in pork meat than in liver at the highest lactic acid concentration. To reach more than 2 logs inactivation, 2.2 M lactic acid is needed. The bacteriophages are inactivated by the most 1 log_{10} at 2.2 M lactic acid.

Regarding models for Hep E virus, if FCV is considered a good model for Hep E virus, this means that in dry ham, a high pressure of 5-600 MPa is needed to achieve a good effect.

The effect seems to depend much on the type of food. The bacteriophages are more stable here than reported for oysters and 6-700 MPa at 20℃ (3), which achieved a complete inactivation of a bacteriophage of the same family as MS2. If HepE virus is more stable than FCV, these models could be relevant.

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TICK-BORNE ENCEPHALITIS OUTBREAKS THROUGH RAW MILK CONSUMPTION IN HUNGARY

Emőke FERENCZI[1, 2], Klára SZÉLES[3], Zsuzsanna BALOGH[1], Wlodzimierz GUT[4]

[1]*National Center for Epidemiology, Department of Virology, Budapest, Hungary;*
[2]*Veterinary Medical Research Institute of Hungarian Academy of Sciences, Budapest, Hungary;*
[3]*National Public Health and Medical Officer Service, Western Transdanubian Regional Institute, Department of Epidemiology, Győr, Hungary;*
[4]*National Institute of Hygiene, Department of Virology, Warsaw, Poland*

Keywords: goat milk, tick-borne encephalitis

Introduction and objectives

The main transmission route of tick-borne encephalitis virus (TBEV) infection is via tick-bite, although the disease can develop following the consumption of raw, non-pasteurized milk of infected animals (goats, sheep, cows), as well [1]. When an infected tick bites the animal host, the infection remains sub-clinical in the animals followed by full recovery but the virus is secreted with milk in low concentrations during the viremic phase. Not only raw milk can be infectious but also the dairy products made of non-pasteurized milk e.g. cheese [2].

In August 2007 a tick-borne encephalitis outbreak took place in Western Hungary, during which 31 people of 154 exposed became ill. All the patients drank raw milk produced by the same goat farm and none of them had tick bite in the anamnesis in the critical time. Beside the laboratory's primary task of examining the samples of patients an extensive investigation of the outbreak was conducted involving the testing of samples from goats and from exposed but asymptomatic people.

Materials and methods

Human samples: serum samples were collected from 31 patients with suspected TBE virus infection and consumption of raw goat milk in the anamnesis. For three patients cerebrospinal fluid samples were also available. Sera from 8 exposed but asymptomatic people were also collected for epidemiological survey.

Animal samples: blood samples were taken from all the goats on the farm that was suspected to be the source of infection.

Samples were examined with several in-house serological methods, namely indirect immunofluorescence assay, hemagglutination inhibition assay and microneutralization assay. A group of the samples were also tested with an ELISA newly developed from a commercially available kit by the Polish partner.

Results

Out of the 39 serum samples sent to the laboratory for serological examination 25 proved to be positive for anti-TBEV antibodies, both IgG and IgM, in IIF tests and also in confirmatory HI assays. Two of the 3 cerebrospinal fluid samples tested positive, the third one negative for specific IgG.

Six patients had symptoms that are characteristic of the first phase of TBE and no second febrile phase and/or neurological symptoms were observed. The sera of these patients tested negative for anti-TBEV antibodies.

Among the 39 serum samples, sera of eight exposed but asymptomatic people were also examined. These people all drank raw milk from the same goat farm, however, antibodies were only detectable in one of these sera, which was probably the result of a previous infection.

As the standard method of human diagnostics, IIF assays were first used to test the goat sera. However, this method gave a highly aspecific positive result for the goat samples, and thus alternative examination methods were applied to determine and confirm the antibody titres, particularly of those samples that had outstandingly high IIF titres. Out of 75 goats, 12 tested positive (11 milked does and one not milked) and 11 doubtful with the HI assay. All the positive and 4 of the doubtful samples were fractionated by chromatography and the IgM fraction was subjected to 2-mercaptoethanol treatment to detect whether reducible IgM is present (shown by titre decrease). Significant titre decrease could be observed in 1 sample.

34 goat serum samples were subjected to microneutralization testing, of which 3 proved to be positive, 4 doubtful and 27 negative for anti-TBEV antibodies. The positive samples were also positive with HI and IIF methods.

The ELISA testing was carried out by the Polish partner. 19 samples were sent to be tested, out of which 2 tested positive, 4 doubtful and 13 negative.

Discussion and conclusions

The National Reference Laboratory for Viral Zoonoses in cooperation with clinicians, veterinarians and epidemiologists extensively investigated the outbreak of 2007. Sera from the goats on the farm were collected to identify the infected animals and a group of exposed but asymptomatic people were asked for blood samples to find possible asymptomatic infections.

Various diagnostic methods were applied for the examination of goat blood samples to ensure the most exact results. The indirect immunofluorescent tests used for routine human diagnostics proved to be highly aspecific when used with goat samples, since nearly all sera were reactive with this method. Hemagglutination inhibition assays gave more reliable and differentiated results when aspecific hemagglutinins and inhibitors were thoroughly removed from the samples. Microneutralization assays and the modified ELISA developed by the Polish partner proved to be very specific and efficient methods for the detection of anti-TBEV antibodies from goat sera, both of them are recommended to use as confirmatory tests in the future.

As the different methods gave varying results, careful consideration preceded the final evaluation of the goat samples. We can surely declare that out of 69 milked goats sera of 2 were positive for anti-TBEV IgG type antibodies with all methods. One of these positive sera also tested positive for TBEV specific IgM, which means that an actually infected goat was identified. This animal may have been the source of infection. The other goats whose sera were IgG-positive in HI and NT and doubtful in ELISA might have experienced an infection previously.

Since the farmers mixed the milk of the goats and sold it that way to the people, we do not know for sure which goat(s) infected which people. It is remarkable, however, that the virus originating from one or a few animals could infect so many people even in a dilution due to the mixing of the milk. This means that raw milk consumption can pose a considerable health risk, particularly in areas where TBE cases frequently occur, i. e. in natural foci where animals are also exposed to tick-bites and consequently to TBEV infection. Western Hungary, where this outbreak was located is the most TBEV-infected part of the country and several earlier milk-borne cases occurred in this area. On the other hand, the 6 asymptomatic people whose blood samples were available for testing did not have anti-TBEV antibodies in their sera, similarly to the 6 patients who did not have second phase symptoms; thus no latent or asymptomatic infection was found in this 12 of the exposed group of people.

Milk-borne infections would be easily avoidable if the milk was pasteurized before consumption, since the heating inactivates the virus. The epidemiological survey revealed, however, that most of the exposed people consumed the raw goat milk because it is widely thought to be an effective natural medicine against certain chronic diseases (allergy, diabetes etc.). As healthy and „bio" lifestyle is getting more and more popular, we should prepare for more people consuming raw milk and dairy products made of non-pasteurized milk.

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MOLECULAR ANALYSIS AND CHARACTERIZATION OF SWINE AND HUMAN INFLUENZA VIRUSES ISOLATED IN HUNGARY IN 2006-07

Péter GYARMATI[1-4*], Giorgi METREVELI[1], Sándor KECSKEMÉTI[2], Mónika RÓZSA[3], Sándor BELÁK[1], István KISS[1, 2]

[1]Departments of Virology, National Veterinary Institute and Swedish Agricultural University,Uppsala, Sweden; [2]Department of Microbiology, Central Agricultural Office, Veterinary Diagnostic Directorate, Bornemissza u. 3-7, H-4031 Debrecen, Hungary; [3]National Center for Epidemiology, Department of Respiratory Viruses, Gyáli u. 2-6, H-1097 Budapest, Hungary; [4]University of Kentucky College of Medicine, Department of Physiology, 138 Leader Ave., 40506-9983Lexington, Kentucky, USA

Keywords: genetic correlation, human, influenza virus, swine

Introduction and objectives

In order to provide additional information to the epidemiological situation in Middle Europe and open further possibilities to investigate the transmission of influenza viruses between species, the viral genomes of three influenza A virus isolates (one human and two swine) collected from North-East Hungary in 2006-07 has been fully sequenced. The sequence analysis reveals strong geographical relationships between the internal genes of the two swine viruses; the human isolate shows strict conservation to recent H1N1 strains, whilst the swine strains demonstrate and reflect a mixed avian-human origin. No evidence of interspecies interaction has been found amongst the studied isolates.

Influenza virus infections and influenza-like illnesses cause substantial economic costs in the human population through loss of working days, hospitalization, epidemic and preventative measures; and because of the zoonotic potential of influenza viruses, there are constant expenses related to the pandemic alert. According to recent assessments, the annual cost of upper respiratory infections is around 80-100 billion USD in the United States of America, and it is not possible to estimate the costs for developing countries, even in the case of extended epidemics (http://www.who.int/mediacentre/factsheets/2003/fs211/en/).

Influenza viruses belong to the Orthomyxoviridae family and are grouped (A, B, C) according to their structural proteins. Influenza A viruses (IAV) are further classified based on the differences in the surface antigens, hemagglutinin (HA) and neuraminidase (NA). Currently 16 HA and 9 NA types are known (1). Birds are natural reservoirs of the virus, having large gene pools with wide varieties of IAV. Swine is considered a transmitter ("mixing vessel") between birds and humans in the evolution of influenza A viruses, playing an important role in the interspecies transmission (2).

The relevance of this topic is shown by the recent swine influenza outbreak started in Mexico, where – until April 29, 2009 – 9 countries have reported 148 cases in less than a week and the human-to-human transmission of swine influenza seems to be proven (further information and update on the situation can be found at: http://www.who.int/csr/disease/swineflu/en/index.html). The presented work reports on the results of whole-genome sequencing and sequence analysis of three influenza A virus strains (two of swine and one of human origin), from the same location and time period (North-East Hungary, 2006-2007). Despite the importance and still not fully understood nature of virus transmission between species, the amount of available data in this region is very limited: according to the NCBI Influenza Virus Resources (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/multiple.cgi), no whole genome IAV sequences are available from Middle Europe, 2006-07. Thus, more sequencing data from that area can help formulation of a complex epidemiological knowledge about the behavior of influenza viruses.

Materials and methods

The A/swine/Hungary/13509/07/H3N2 (H3N2sw) strain was collected in the summer of 2007 from Kunhegyes, Hungary, the A/swine/Hungary/19774/06/H1N1 (H1N1sw) strain was collected in the late autumn of 2006 from Kaba, Hungary, and the A/Nyiregyhaza/01/2007/H1N1 (H1N1hu) strain was collected in, 2007 January from Nyiregyhaza, Hungary. All viruses were isolated by using 10 days old embryonated chicken eggs. Subtypes were determined by hemagglutination inhibition and by padlock probes (3), with identical results. RNA-extraction, RT-PCR amplification, sequencing and analysis of the sequences were performed as described previously (4).

Phylogenetic analysis and molecular characterization.

For comparison, 35 full-length IAV sequences were selected from the Influenza Virus Resources, representing swine and human influenza strains with different geographical origins. Sequences were aligned using Clustal W (5) and cut to equal lengths with the CLC Combined Workbench v3.0.2. (CLC bio A/S, Aarhus, Denmark) Distance based phylogenetic trees were constructed using the MEGA v4.0 software (6) with 1000 replicates; the results were verified by maximum likelihood trees created by the fastDNAml program, as part of the BioEdit Sequence Alignment Editor software (v7.0.5.2) (7). The pairwise alignment comparisons were also performed by using the BioEdit Sequence Alignment Editor software.

Discussion and conclusions

The importance of sequencing of influenza genomes is reflected in the current Mexican outbreak of swine influenza, because the proof of origin is still the – at least partial – sequencing.

The purpose of this study was to acquire more information about the influenza strains and their possible relations circulating in Hungary. The presented work describes the analysis of 3 whole genome influenza sequences isolated in North-East Hungary in 2006-07. Since no full-length IAV sequences have been reported from this region so far, a secondary aim of this work was to obtain epidemiological information about the circulating virus strains by comparison with isolates from abroad. The aspects of selection of samples were based on the similar mammalian origin, time period, and represented by different geographical origins (including the major swine lineages), involving recent European and "classical" American and Asian swine H1N1 viruses (23). Swine influenza outbreaks are rare at the region of the study (the last diagnosed epidemic in the same area was reported in 1992) but provided a good occasion to obtain recent swine influenza virus strains and get an insight into their molecular characteristics and their possible relationship to a contemporaneous human isolate. The one human and the two swine viruses were isolated within one year.

Though it is unlikely that three strains can represent the entire population of influenza viruses in pigs and humans in a country, it should be considered that human H1N1 viruses show high level of conservation, and the occurrence of swine influenza is low and cases are rarely reported (e.g., due to quick progress, asymptomatic infections, or the high morbidity but low mortality (2, 22).

The comparative genomic analysis of the three strains reveals strong geographical relatedness to other European isolates, but the composition – especially the H3N2sw isolate – shows divergence and might reveal a more complex origin. As expected, surface proteins do not obviously follow the same pattern as the structural proteins because of the subtype determination, but the high level of similarity between the structural genes of swine isolates emerge the possibility of their common origin; while there is no direct relation shown with the human strain. However, the studied H1N1 isolates possess similar patterns in the polymerase and non-structural genes, associated with host adaptation processes. Surprisingly, a Chinese swine isolate (also from 2007) shows high similarity to Hungarian swine isolates, which might arises a trade-related issue, which has to be considered as an important factor in influenza control and eradication in the intensified international trade.

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MOLECULAR DETECTION OF KOBUVIRUSES AND RECOMBINANT NOROVIRUSES IN CATTLE IN CONTINENTAL EUROPE

Axel MAUROY, Alexandra SCIPIONI, Elisabeth MATHIJS, Christine THYS, Etienne THIRY

Virology and Viral Diseases, Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, Liège, Belgium

Keywords: cattle, epidemiology, kobuvirus, norovirus, PCR, recombinant

Introduction and objectives

Noroviruses (NoV) and Kobuviruses (KoV), belong to the family *Caliciviridae* genus *Norovirus* and to the family *Picornaviridae* genus *Kobuvirus* respectively. Both have a single stranded positive-sense RNA genome. They both infect the gastrointestinal tract of different animal species including human beings. Two NoV and one KoV prototype strains have been already identified in the bovine (Bo) species: Jena virus (JV) and Newbury 2 (NB2) for BoNoV; U1 for BoKoV. Genogroup (G) III gathers all BoNoV strains and is further subdivided into two genotypes where viruses genetically related to JV and NB2 are assigned to the genotype 1 and 2 respectively. Recombination is a common event in NoV and is usually reported near the overlapping region between open reading frame (ORF) 1 (end of the polymerase gene) and ORF2 (beginning of the single capsid protein gene). Two GIII.1/GIII.2 BoNoV recombinant strains have been described including the recombinant strain Bo/NoV/Thirsk10/00/UK (Thirsk10), identified in the year 2000 in Great Britain. To our knowledge, no other genetically related strains have been reported since [1]. Bovine KoV were detected by RT-PCR in stool samples of healthy calves from Japan, in samples from diarrhoeic calves from Thailand [2] and were also identified very recently in Hungary. Bovine NoV prevalence studies performed in different areas have shown the predominance of the GIII.2 genotype but this could reflect a GIII.1 specificity failure in the RT-PCR methods. The aim of this study was to screen cattle stool samples with two primer sets targeting the polymerase and the capsid region. The primer pair targeting the capsid region was designed based on a GIII.1 sequence in order to improve their detection.

Materials and methods

A stool bank (n=300) was created with calf and young stock diarrhoeic samples from five provinces in Belgium (Hainaut, Liège, Namur, Luxembourg, Walloon Brabant) and received from a Belgian diagnostic laboratory through the year 2008. Viral RNA extraction was performed and one step RT-PCR was carried out on 2 µl of each viral RNA extraction using the CBECu-F/R primers (nucleotidic position on JV: 4543-4565 and 5051-5074) and a primer pair, named AMG1-F/R, designed from the JV genomic sequence (F: tgtgggaaggtagtcgcgaca, nucleotidic position on JV: 5012-5032; R: cacatgggggaactgagtggc, 5462-5482). Combined approaches with the CBECu-F and AMG1-R primers, additional internal primers (F2: atgatgccagaggtttcca, position on JV: 4727-4745; R2: gcaaaaatccatgggtcaat, 5193-5211) or CBECu-F and a polyTVN-linker were also carried out on some positive samples. RT-PCR products were directly sequenced twice or cloned before sequencing. Sequencing was carried out at the GIGA facilities of the University of Liège with BigDye terminator kit. Nucleotidic sequences were analysed with the BioEdit software. Nucleotidic similarity with the NCBI genetic database was assessed using the BLAST tool. Phylogenetic inference was performed with the MEGA software. Phylogenetic tree was constructed by neighbour-joining analysis where evolutionary distances were computed using the Maximum Composite Likelihood method. The confidence values of the internal nodes were calculated by performing 1,000 replicate bootstrap values. Genetic recombination was analysed with the Simplot software and the Recombinant Detection Program.

Results

Twenty-eight positive samples were identified in the 300 samples: 24 and 23 BoNoV sequences with the CBECu and AMG1 primer pairs respectively, giving a combined apparent molecular prevalence of 9.33% (CI 95%: [9.27; 9.38%]). Using BLAST, three sequences amplified with CBECu-F/R (BV164, BV362, and BV416) were genetically more related to the GIII.1 JV and Aba Z5/02/HUN sequences and one (BV168) to the recombinant strain Thirsk10. The others were genetically related to GIII.2 BoNoV. All the sequences amplified with AMG1-F/R but one genetically matched with GIII.2 BoNoV. The AMG1-amplicon of the BV416 sample matched with the recombinant strain Thirsk10. A 2410 nucleotide (nt)-large genomic sequence was obtained from BV416 with CBECu-F/TVN linker, which was a recombinant sequence genetically related to the Thirsk10 strain. This result was confirmed by phylogenetic and by Simplot analysis. The potential recombination breakpoint of BV416 was located near or within the ORF1/ORF2 overlapping region depending on the bioinformatic program used. Comparison between its different genomic regions and the JV, Newbury2 and Thirsk10 genomic sequences showed that the polymerase region of BV416 was genetically more related to the GIII.1 than to the recombinant strain. F2/R2 amplicons from BV164 and BV362 were genetically related to GIII.2 and GIII.1 BoNoV respectively. Surprisingly, three amplicons obtained with the combined primer pair CBECu-F/AMG1-R on BoNoV positive samples at the expected molecular weight did not match genetically with BoNoV but did so with different genomic regions of the BoKoV U1 strain (86%, 92% and 93% of nucleotidic identity by BLAST for BV228, 250 and 253 respectively on sequences of about 500-700 nt).

Discussion and conclusions

In this study, very few genotype 1 BoNoV were identified (BV362 was the sole GIII.1 sequence obtained in the ORF1/2 overlapping region), confirming results reported in a previous study on BoNoV infection in the same area [3]. A recombinant status was clarified for BV416. Co-infection with GIII.1 and GIII.2 BoNoV was evidenced in the BV164 sample but could not be excluded in the BV168 sample because an overlapping sequence could not be obtained, although genetic analyses related its CBECu-F/R sequence to the Thirsk10 sequence. These results raise issues about the genetic characterization by primers targeting either the polymerase region or the capsid region. By exclusion of the potential recombination breakpoint, these primers can lead to the misclassification of strains and to the underestimation of circulation of recombinant strains. Multiple alignment and bioinformatic analysis performed with JV, Aba Z5, NB2, Thirk10 and BV416 sequences has suggested a recombination breakpoint for BV416 located near the ORF1/ORF2 overlapping region and one quite similar to those determined for the Thirsk10 strain. Nevertheless the greater similarity of BV416 with the Jena and Aba Z5 viruses in the polymerase region and the exact localization of the recombination breakpoint suggest another origin or genetic evolution than the Thirsk10 strain. The identification, in geographically and temporally different samples, of sequences that could be genetically related to the recombinant Thirsk10 strain suggests at least that Thirsk10-related strains circulate in the north European cattle population. Furthermore, the low detection rate of GIII.1 BoNoV could reflect an evolution of the viral population pattern to the benefit of the Thirsk10-related and genotype 2 strains in the studied region. To date, BoKoV-related sequences have been very rarely identified, and in only three countries (namely Japan, Thailand and Hungary). Their detection in another European country suggests their wider distribution, making them at least emerging bovine viruses in the studied region. In conclusion, prevalence studies on BoNoV using RT-PCR assays, even targeting relatively well conserved genomic regions, need to take into account in their protocols both their high genetic variability and their relative genetic proximity with other viruses, in order to maximize sensitivity and specificity. This study also showed that recombination events could lead to emerging strains in the BoNoV population, as already found for HuNoV. The molecular detection of bovine kobuvirus-related sequences in the studied area extends the distribution of these viruses in Europe.

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GENETIC CHARACTERISATION OF H9N2 VIRUSES ISOLATED FROM THE KINGDOM OF SAUDI ARABIA

Isabella MONNE[1], Alice FUSARO[1], Mahmoud M. ISMAIL [2], Mohamed H. AL-BLOWI [3], Owais A. KHAN[4], Annalisa SALVIATO[1], Alessia SCHIVO[1], Ilaria CAPUA[1], Giovanni CATTOLI[1]

[1]*Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padova, Italy;*
[2]*Central Veterinary Diagnostic Laboratory, Riyadh 11454, KSA. and Department of Poultry Diseases, College of Veterinary Medicine, Kafr-Elsheikh University;*
[3]*Veterinary Labs Administration, Ministry of Agriculture, Riyadh, KSA;*
[4]*Animal Diagnostic Laboratory, Pennsylvania State University, University Park, PA, USA*

Keywords: H9N2, influenza A virus, Kingdom of Saudi Arabia

Introduction and objectives
Since the late 1990's avian influenza viruses of the H9N2 subtype have become prevalent in domestic poultry in many countries in Asia and the Middle East. Despite being LPAI viruses, H9N2 viruses have raised concerns for high morbidity and mortality caused in poultry and for sporadic cases of human infection. In the Kingdom of Saudi Arabia (KSA) the first outbreak in poultry was reported in 1998. Since then, several H9N2 outbreaks have occurred. Furthermore, the KSA poultry industry has recently experienced infection with H5N1 highly pathogenic avian influenza (HPAI) viruses (2). The co-circulation of distinct avian influenza subtypes has increased the likelihood of generation of novel reassortant viruses with unknown zoonotic potential. Little is known about the gene constellation of the H9N2 viruses that are circulating in KSA. The objective of this study was to analyse the genetic characteristics of nine H9N2 Saudi Arabian viruses isolated from different bird species between 2005 and 2007.

Materials and methods
Nine influenza A viruses of the H9N2 subtype isolated from poultry in Saudi Arabia between 2005 and 2007 were used in this study. All viruses were grown in 9-to-10-day-old embryonated fowls' specific pathogen free (SPF) eggs. Subtype identification of the viruses was determined by standard hemagglutination inhibition test, as described in European Union Council Directive 2005/94/EC and the neuraminidase inhibition test. Subsequently the whole genome of each of nine H9N2 isolates was sequenced.

The amplification of the 8 viral gene segments was done by RT-PCR using gene-specific primers (available upon request). PCR products were purified (ExoSAP-IT, USB, Cleveland, Ohio, USA) and sequenced in a 3130xl Genetic Analyser (Applied Biosystems). Phylogenetic analysis was performed using the neighbour-joining method in the MEGA 3 programme. The HA and NA tree topology was confirmed using Bayesian methods. To analyse amino acid composition, the 8 gene segment were aligned and translated.

Results
The results of the phylogenetic analysis showed that all the sequences of the HA and NA gene of the KSA viruses clustered in the G1-like lineage previously described by Xu et al, 2005 (5). This lineage comprises human H9N2 isolates from Hong Kong and several H9N2 viruses from Asian and Middle Eastern countries. Within the G1-lineage, some sub-clustering was evident for the HA and NA sequences of the H9N2 viruses analysed (figure 1). For the HA gene 8 out of 9 viruses showed close identity with recent viruses from Israel and Pakistan (similarity between group ranged from 94.8% to 95.3%). One KSA isolate (A/quail/Saudi Arabia/1178-46/2006) resulted slightly divergent from these viruses (similarity ranged from 87.9% to 89.2%) and showed the highest similarity (94%) with the virus A/chicken/Israel/90658/2000.

Analysis of the deduced amino acid sequences of the envelope glycoproteins and the internal genes of the KSA strains revealed molecular markers that confer unique properties, such as a wider host range. Of particular interest is the identification, in 3 out of 9 Saudi Arabian viruses, of one PB2 mutation (A199S) which is 100% conserved in the viruses responsible of 1918, 1957 and 1968 pandemics. Eight Saudi Arabian viruses presented the substitution Q226L (H3 numbering) at the receptor binding site of the HA gene. This mutation is associated with a preferential receptor binding specificity for sialic acid α2,6-linked galactose and with a more efficient replication in human cells in culture (4). The strain A/quail/Saudi Arabia/1178-46/2006 did not possess the leucin at the position 226. However this isolate from quail exhibits 3 out of 4 amino acid residues at the RBS of the HA1 gene previously associated with respiratory transmission in ferrets (3).

Discussion and conclusions
The results of the analysis of the surface glycoprotein genes suggest that at least two H9N2 sublineages can be recognised within the major G1-like lineage in the Kingdom of Saudi Arabia. The analysis performed on the sequences of the Saudi Arabian viruses allowed us to identify specific amino acid substitutions which are believed to modify the efficacy of the replication in non-avian species. In previous studies it has been suggested that quails may represent a suitable host for the generation of avian influenza strains capable to replicate more efficiently in mammalian hosts (1). In this study an H9N2 quail isolate, with mutations demonstrated to be crucial to support transmission between ferrets, was identified. This finding underlines that quails have to be considered as a strategic target of avian influenza monitoring in the Kingdom of Saudi Arabia and highlights the importance of whole genome analysis to follow the molecular evolution of avian influenza viruses. Considering the high prevalence of H9N2 viruses throughout the Middle East along with the ability of some H9 strains of the G1 lineage to infect humans more investigation must be undertaken to monitor the evolution of H9N2 subtype in these countries.

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SURVEILLANCE OF EMERGING AND/OR NEGLECTED PATHOGENS IN PIGEONS IN URBAN AREAS

Marta PÉREZ-SANCHO[1], Julio ÁLVAREZ[1], Nerea GARCÍA[1], Irene MARTÍNEZ[1], Sergio GONZÁLEZ[1], Pilar LIÉBANA[1], Joaquín GOYACHE[1, 2], Francisco MENA[3], Lucas DOMÍNGUEZ[1, 2]

[1]Centro de Vigilancia Sanitaria Veterinaria-VISAVET, Universidad Complutense de Madrid, Madrid, España
[2]Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, España;
[3]Departamento de Sanidad y Consumo, Ayuntamiento de Alicante, Alicante, España.

Keywords: emerging pathogen, neglected pathogen, PCR, peridomestic animal

Introduction and objectives
At least 61% of all human pathogens are zoonotic, and have represented 75% of all emerging pathogens during the past decade (WHO, 2009).The vast majority are not prioritized by health systems at national and international levels and are therefore labelled as neglected (WHO, 2009). Several species of peridomestic animals are susceptible to these pathogens, therefore becoming potential sources of infection for humans. Among them, fereal pigeons, which have colonized cities in Europe and live in close contact with humans and other animals, could become a significant focus of infection. In this sense, they can harbor more than 50 different human pathogenic organisms. For this reason, a study to evaluate the presence of emerging and/or neglected zoonotic pathogens [West Nile Virus (WNV), Avian Influenza Virus (AIV), Newcastle Virus (NCV) and *Chlamydophila psittaci*] was carried out to assess the potential role of these urban birds in the epidemiology of these agents in the city of Alicante, in the eastern coast of Spain.

Materials and methods
A total of 50 pigeons of different sex and age were trapped using nets in the city of Alicante during the spring of 2008. Animals were transported in adequate conditions to VISAVET Health Surveillance Centre (UCM, Madrid, Spain) where they were euthanasied by carbon dioxide inhalation immediately upon arrival. Subsequently, birds were necropsied and examined for the presence and location of macroscopic lesions. Cloacal and tracheal swabs were collected from all pigeons. Pooled swabs (n=5) were subjected to DNA/RNA extraction protocols in Physical Containment Level 3 Laboratory using QIAamp Viral RNA mini kit (QIAGEN) according to manufacturer's instructions. Detection and identification of each pathogen was performed using molecular techniques. Specific Polymerase Chain Reaction (PCR) was utilized to detect WNV, NCV and *C. psittaci*. AIV was detected through one step Real Time PCR. Statistical analysis of the data was carried out using software EPI 4.0 (Computer Program for Epidemiologist).

Results
No macroscopic lesions were observed during the necropsy. No positive results were found for neither Avian Influenza virus nor West Nile virus. One pool was positive at NCV PCR analysis, yielding an apparent prevalence of 2,09% (95% C.I., 0.05-11.10%). This positive result was confirmed by sequencing of the amplicon obtained after amplification of the matrix gene. *C.psittaci* analysis revealed an apparent prevalence of 9.71% (95% C.I., 2.56- 23.48%) since four pools were positive. No difference of results depending on age or sex of analyzed animals was observed in our study.

Discussion and conclusions
Our data suggest that pigeons can be carriers of important emerging and/or neglected pathogens and could play a significant part in the epidemiology of these agents. Neither AIV nor WNV presence were detected, although other studies have described Newcastle disease and Avian Influenza as the most important viral diseases that could be transmitted to poultry farms by free-living pigeons (Dovc *et al.,* 2004). Moreover, potential role of these birds in amplifying WNV in urban settings has been suggested (Allison *et al*., 2004). In our case, the low number of tested animals, and the use of pools instead of single samples could explain the absence of positive results. Our results regarding presence of NCV in Alicante are in accordance with several studies that confirmed NCV circulation among pigeon population in other cities by serologic detection (Tshai *et al*., 2006). At last, apparent prevalence of *C. psittaci* found in our study is in agreement with previous reports in other cities. Our results confirm that *C. psittaci* is one of the most widespread zoonotic pathogens in feral pigeons population (Haag-Wackernagel and Moch, 2004).

In summary, the results of this study demonstrate that pigeons may constitute a reservoir for important pathogens which may suppose a risk to other animals and humans. Detection of neglected pathogens using peridomestic animals as sentinels is an affordable way of monitoring presence of these microorganisms in urban areas. Although further studies with larger number of animals should be carried out in order to determine the role in transmission and maintenance of emerging and/or neglected agents posed by these animals with more accuracy, performance of surveillance programs for detection of these microorganisms on peridomestic animals like free-living pigeons is a cost-efficient way for demonstration of their presence in human settings.

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DESIGN OF AN ELISA TEST SPECIFIC FOR GENOTYPE 3 HEPATITIS VIRUS E IN SWINE.

Nicolas ROSE[1], Annie BOUTROUILLE[2], Audrey FRAISSE[2], Aurelie LUNAZZI[2], Marc ELOIT[2], Nicolas PAVIO[2]

[1]Unité d'Epidémiologie et Bien-Etre du Porc, AFSSA, Ploufragan.
[2]UMR 1161 Virologie AFSSA LERPAZ, ENVA, INRA, Ecole Nationale Vétérinaire d'Alfort, Maisons-Alfort., France

Keywords: ELISA, hepatitis

Introduction and objectives
Hepatitis E virus (HEV) is divided into four genotypes and so far, only one serotype has been described. In endemic regions, serotypes 1 and 2 are mainly found in human population. In non-endemic regions, such as Europe, USA and Japan the situation is different and genotype 3 is found in both human autochthonous cases and animals (swine, wild boar or deer). HEV serological diagnosis is based on recombinant antigens or peptides derived from genotype 1 and 2. Considering HEV genotype 3 diversity (10 subtypes) and quasi-species it is possible that serotype divergence might impair accurate serodiagnosis. Furthermore, since swine is a possible reservoir for human infection, it is necessary to develop a serological test specific of swine strains. Thus, we have engineered a recombinant baculovirus expressing a capsid protein of a swine genotype 3 HEV.

Materials and methods
HEV ORF2 was purified from the supernatant of SF9 cells infected with a recombinant baculovirus. This protein self-assembles into "Virus Like Particles" (VLPs). VLPs were directly coated onto polystyrene ELISA plates and after incubation with swine sera, bound HEV antibodies were detected with a rabbit anti-porcine IgG HRP-conjugated polyclonal antibody which was then revealed using a TMB-substrate.

Results
Estimation of sensitivity and specificity was performed using sera from experimentally HEV genotype 3 infected pigs and negative sera from SPF pigs. Further validation of the assay was performed using sera from 34 independent herds collected at slaughter house. All pig sera were tested using the VLPs based genotype 3 assay in comparison with a commercial serologic assays based on genotype 1 & 2 antigens. HEV seroconversion in pigs infected with HEV genotype 3 virus was detected only with the genotype 3 VLPs test. Among the 32 SPF pig serum tested none were positive with both tests. Similar prevalence within the 34 herds were observed using both test (73 and 76%) but higher prevalence within positive herds were observed using the genotype 3 VLPs based assay. For HEV antibody testing no gold standard is available thus a latent-class Bayesian approach for correlated tests was used to estimate the sensitivity and specificity of both tests. Prior distributions for prevalence, sensitivities, specificities were determined using Beta distributions because they are related to the Binomial distribution and give a good representation of this kind of biological data (unimodal distribution bounded within [0, 1] interval, possibly skewed). Parameters of Beta distributions were determined using previous data (for Prevalence, based on the available results from a current survey) or from expert elicitation for sensitivity and specificity. Mildly informative priors were taken for prevalence at the individual level and sensitivity of both tests (they were assumed to be >0.6 with a mode at 0.9 with 95% certainty). More informative priors were taken for specificity as both tests gave negative results with samples taken from SPF animals, known to be negative for VHE. Thus specificities were assumed to be >0.95 with mode at 0.99 with 95% certainty. Analyses were carried out using Winbugs software. The mean sensitivity of the commercial test was estimated to be 0.47 with 95% credibility interval being [0.39-0.55] whereas the mean sensitivity of the VLPs based genotype 3 assay was estimated to be 0.92 [0.81-0.99]. Specificities were estimated to be 0.98 [0.93-0.99] and 0.98 [0.95-0.99] for the VLPs-based and commercial tests respectively.

Discussion and conclusions
VLPs derived from swine HEV genotype 3 are good candidates for the improvement of hepatitis E serology in non endemic region and particularly into pigs. It is thus of major interest for a zoonotic agent with possible transmissions to human through food products.

FOUR DIFFERENT SUBLINEAGES OF HIGHLY PATHOGENIC AVIAN INFLUENZA H5N1 INTRODUCED IN HUNGARY IN 2006-2007

Zsófia SZELECZKY[1], Ádám DÁN[1], Krisztina URSU[1], Éva IVANICS[1], István KISS[2], Károly ERDÉLYI[1], Sándor BELÁK[2], Claude P. MULLER[3], Ian H. BROWN[4], Ádám BÁLINT[1]

[1]Department of Virology, Central Agricultural Office Veterinary Diagnostic Directorate, Tábornok u. 2, H-1581 Budapest, Hungary
[2]The Joint Research and Development Division, in Virology of the National Veterinary Institute (SVA) and Swedish University of Agricultural Sciences (SLU) and Department of Biomedical Sciences and Public Health, Section of Parasitology and Virology, SLU, Ulls väg 2B, SE-75189 Uppsala, Sweden
[3]Institute of Immunology, National Public Health Laboratory, 20A rue Auguste Lumiere, L-1950 Luxembourg, Luxembourg
[4]Virology Department, Veterinary Laboratories Agency-Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom

Keywords: avian influenza, H5N1, highly pathogenic, Hungary, phylogenetic characterisation

Introduction and objectives

In Hungary, the first wave of HPAI H5N1 outbreaks occurred in wild birds in February-March 2006 (3). The virus temporarily disappeared from the territory of Hungary, but unexpectedly re-emerged in June 2006 and this second wave caused the largest domestic waterfowl epizootic in Europe (1). The third wave of outbreaks of HPAI H5N1 in domestic geese was reported in January 2007.

Since H5N1 affected Hungary in three separate waves in 2006/07, this study presents the full-length genomic coding regions of the index strains of these epizootics in order to; (i) understand the phylogenetic relationship to other European H5N1 isolates, (ii) elucidate the possible connection between the different outbreaks and (iii) determine the putative origin and the way of route of introduction of the different virus variants.

Materials and methods

The viruses were isolated from dead wild waterfowl, domestic goose, duck and guinea fowl according to European standardised protocols. Viral RNA was extracted from infective allantoic fluid by High Pure Viral RNA Extraction Kit (Roche Applied Science). For amplification of the whole coding sequence of the viral genes, a previously published protocol (2) was applied. The amplicons were generated with the Qiagen OneStep RT-PCR Kit (QIAGEN). The amplicons were sequenced with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an automatic ABI PRISM 3130 genetic analyser (Applied Biosystems). Sequences were assembled and edited using the BioEdit v.7.0.7 and the DNASTAR 7 (Lasergene) software package. Genotyping of the gene segments were carried out with Influenza A Virus Genotype Tool. Distance based neighbour-joining and character based maximum parsimony phylogenetic trees were generated using the Molecular Evolutionary Genetics Analysis (MEGA) software v.4.0. The topology of trees was confirmed by 1000 bootstrap replicates. Maximum likelihood phylogenetic trees were generated by the PHYLIP version 3.68 software. Trees were edited with Tree Explorer program of MEGA v.4.0. For phylogenetis analysis, in addition to the four full-length coding sequences of the Hungarian isolates, a set of H5N1 strains from Europe, Middle-East, Africa and Asia were selected.The sequences were deposited in GenBank under the following accession numbers: FJ445226-FJ445249.

Results

During the course of the epizootics, all HPAI H5N1 viruses isolated from the 64 wild bird and 31 domestic poultry cases were subjected to partial molecular analysis. The obtained preliminary sequence data showed that the wild bird isolates can be divided into two distinct sublineages designated Hungarian1 (HUN1) and Hungarian2 (HUN2) groups (4).

In the present study 12 wild bird H5N1 isolates were selected for further genetic analysis based on their geographical origin. The HUN1 and HUN2 index strains, A/mute swan/Hungary/3472/2006 and A/mute/swanl/Hungary/4571/2006 were subjected to complete genome analysis, whereas the HA coding sequences were determined from the remaining ten strains. Molecular analysis of the HA gene of the wild bird isolates confirmed confirmed the previous HUN1 and HUN2 clustering corresponding to sublineage 2.2B and clade 2.2.1, respectively. Sequencing the whole coding region of the two index viruses of thes e groups suggests the role of wild birds in the introduction of HUN1 and HUN2 viruses: the most similar isolates to HUN1 and HUN2 group were found in wild avian species in Croatia and Slovakia, respectively.

The second introduction of HPAI H5N1 led to the largest epizootic in domestic waterfowl in Europe affecting 29 goose and duck holdngs, and one million birds were culled during the implementation of control measures. The index strain of the epizootic A/goose/Hungary/14756/2006 clustered to sublineage 2.2.A1 forming the Hungarian3 (HUN3) group. A common ancestry of HUN3 isolates with Bavarian strains is suggested as the most likely scenario of origin. Hungarian4 (HUN4) viruses isolated from the third introduction formed a monophyletic cluster with isolate A/turkey/United Kingdom/750/2007 forming a sublineage 2.2.A2.

Discussion and conclusions

Since genetic databases of European, Middle East and African H5N1 strains have expanded dramatically recently, positioning Hungarian isolates in phylogenetic trees of relatively high resolution is an invaluable genetic tool to determine their putative origin.

Molecular analysis of the HA gene of HUN1 and HUN2 wild bird HPAI strains revealed equences within the groups were in most cases identical, which phenomenon can be explained by the restricted geographical area and time course of these outbreaks. Sequencing of the whole coding sequence of the geographically distinct two index viruses A/mute swan/Hungary/3472/2006 and A/mute swan/4571/Hungary/2006 revealed that each segment clustered with the corresponding clusters indicating the absence of segment reassortment.

HUN1 viruses emerged at the beginning of February 2006 in Southern Hungary in the wetland areas along river Danube. Interestingly, during the course of the wild bird outbreaks, the HUN1 viruses remained restricted to this small geographical area.

HUN2 viruses were detected ten days after the HUN1 strains in Northern Hungary. This virus variant spread southward along the line of river Danube to the area where HUN1 strains were already present. The different virus variants co-existed in the southern part of Hungary for several weeks providing an opportunity for potential reassortment.

The HUN3 strains originated from the second introduction wave of H5N1 may have a common ancestor with the Bavarian strains that emerged some days later than representatives of HUN2 sublineage, and interestingly their distribution was restricted only to Central Europe. The origin and way of introduction of HUN3 strains need further investigation.

The third introduction wave of HPAI H5N1 into the territory of Hungary occurred in January 2007, when no presence of HPAI was reported in the country. A direct epidemiological link was established between the Hungarian and United Kingdom outbreaks. The common ancestor, origin and way of introduction of HUN4 viruses are still obscure.

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INVESTIGATING THE PRESENCE OF INFECTIOUS HAEMATOPOIETIC NECROSIS (IHN) AND INFECTIOUS PANCREATIC NECROSIS (IPN) VIRUS INFECTIONS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM, 1792)

Harun ALBAYRAK[1], Emre OZAN[1],Yuksel DURMAZ[2]

[1]Veterinary Control and Research Institute, Virology Laboratory, Samsun, Turkey;
[2]Veterinary Control and Research Institute, Fish Diseases Laboratory, Samsun, Turkey

Keywords: Ag-ELISA, IHN, IPN, turkey

Introduction and objectives

Infectious hematopoietic necrosis (IHN) virus and infectious pancreatic necrosis (IPN) virus are both important disease agents in farmed rainbow trout, and disease outbreaks cause major losses in fish farming worldwide. IHNV belongs to the new genus Novirhabdovirus of the family Rhabdoviridae. Infectious pancreatic necrosis virus (IPNV) belonging to the Birnaviridae. It is a bi-segmented dsRNA virus which occurs primarily in freshwater, but appears to be saltwater tolerant.

The aim of this study is to determine presence of IHNV and IPNV in fresh water farmed rainbow trout in the Blacksea region in Turkey and to assess the diagnostic values of virus isolation, Ag-ELISA and RT-PCR techniques in diagnosis of these infections.

Materials and methods

Total of 229 isolation material from 168 portion rainbow trout of various sizes (200 to 500 g) and 61 immature fish (from 305 immature fish, weighing 3-10 g) were obtained from 32 farms in the region in december and march in 2006-2007 which period is water temperature under the 12 ^{0}C. All isolation materials were passaged in BF-2 (Bluegill fry-2) cell cultures for two times. While cell culture supernatants showed cytopathic effect (CPE) were tested by antigen-capture enzyme linked immunosorbent assay (Ag-ELISA), all isolation materials were tested by reverse transcriptase polymerase cahin reaction (RT-PCR).

Results

As a result, while IPN virus was detected in 10 farms in Ordu, Samsun, Tokat and Trabzon provinces, IHN virus was not detected. That cell culture isolation and RT-PCR test are harmonious were revealed.

Discussion and conclusions

Production of rainbow trout has an important place in fishery industry in Turkey. Rainbow trout production produced inland waters account for 55,7% in total fisherier inland waters in Turkey. Blacksea Region is the most important place in Turkey with respect to production of natural and cultured fish in both fresh water and marine. Especially, production of rainbow trout in inland waters are done intensely. In this study, IPNV was detected in 10 of 32 fish farms (%44.44), but no IHNV was detected in the same fish farms. In accordance with these results, IPNV infection is widespread in the Blacksea Region.

Acknowledgements

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RESPIRATORY DISEASE ASSOCIATED WITH BOVINE CORONAVIRUS IN ADULT CATTLE IN NORTHERN PORTUGAL

Ana Cristina ARAÚJO, Eliane SILVA, Alexandra MULLER, Gertrude THOMPSON

Institute of Biomedical Science Abel Salazar (ICBAS), Department of Veterinary clinics, University of Porto, Porto, Portugal
Multidisciplinary Unit of Biomedical Investigation (UMIB), University of Porto, Porto, Portugal

Keywords: bovine respiratory disease, coronavirus, genetic variation, respiratory syncytial virus

Introduction and objectives

Bovine Coronavirus (BCoV) was identified as an emerging pathogen of respiratory tract disease in cattle, as it had previously only been reported in enteric disease (Liu et al., 2004; Storz et al., 2000). The virus can spread to animals of all ages causing high morbidity, but usually low mortality (Liu et al., 2004). In the USA, BCoV is considered one of the most prevalent agents in shipping fever (Storz et al., 2000). The same virus strain can be responsible for the simultaneous appearance of enteric and respiratory disease in the same animals (Chouljenko, et al, 2001). The dairy industry in Portugal is concentrated in the North and respiratory disease is responsible for severe economic losses, however information on BCoV infections is scarce. The objectives of this work were to assess the importance of BCoV in dairy cattle with respiratory disease and to genetically characterize any field strains.

Material and methods

Samples of nasal discharge, faecal and whole blood were collected from 29 animals with clinical signs of respiratory disease in dairy herds in northern Portugal. The samples were analysed by PCR for the following four viruses: BCoV, BRSV, BVDV and BHV-1. For the genetic characterization of BCoV, the PCR products – located within the hemagglutinin-esterase (HE) gene - were directly sequenced using the internal primers of the nested PCR (Liu et al., 2004). The obtained sequences of 322 nucleotides spanned positions 23210-23531. Sequences were assembled and analyzed using the software package Bioedit and MEGA3.1. The obtained sequences were compared with published homologues from GenBank database (NCBI).

Results

Of the 29 animals tested by PCR, BRSV was detected in 5 animals (3 in nasal discharge, 2 in blood), BVDV in nasal discharge of 2 animals and BHV-1 in none. BCoV was detected in five samples of 3 animals. BCoV was detected in two calves with respiratory and enteric symptoms simultaneously in nasal discharge and faeces. The nucleotide sequences of these 4 samples were identical (GenBank reference EU703816). They displayed highest similarity with the sequences obtained from wild life (eg. Water buffalo calf, GenBank reference EU019216). The following three polymorphic nucleotide positions were observed between both strains (EU703816 and EU019216, respectively): C23229T, C23310T and A23542C. The highest nucleotide similarity with a BCoV strain from bovine origin was observed with the Italian strain "339/06" (GenBank reference EF445634), in which an additional, fourth nucleotide polymorphism was observed at position C23289T. None of the four mutations resulted in aminoacid changes of the deduced protein. The other positive animal was a cow of a different herd, that at the time of sampling showed dyspnoea, fever (39,5ºC), seromucous nasal discharge without evidence of diarrhoea. BCoV was detected only in nasal discharge but not in faeces. Interestingly, this cow was also positive for BRSV in blood but not in nasal discharge. The BCoV sequencing (GenBank reference EU703815) displayed 100% nucleotide identity with prototype enteric strains such as Kakegawa, Quebec and Mebus (GenBank references AB354579, AF220295 and BCU00735, respectively). The sequence comparison between the strains obtained from these two dairy herds displayed 11 nucleotide polymorphisms, which resulted in two aminoacid changes of the HE protein (EU703815 and EU703816, respectively) at positions S367P and L392I.

Discussion and conclusions

This study has shown that BCoV is present in respiratory tract infections and enteric disease in dairy herds in Portugal. BCoV was detected in 3 of 29 animals with respiratory tract infection. To our knowledge this is the first partial genetic characterisation of BCoV field strains in this country. No differences were found in the partial HE gene sequences obtained from nasal versus enteric BCoV from the two calves of the same herd. Interestingly however, in comparison with the BCoV sequence obtained from a cow of a different herd, 9 synonymous and 2 nonsynonymous nucleotide polymorphisms were observed. Although the HE gene is considered relatively conserved (Liu et al. 2004). our results suggest that considerable variability may exist in the field. The S gene is less conserved and thus a common target for the genetic characterisation and phylogenetic analyses of BCoV isolates (Liu et al. 2006; Liu et al., 2004). Currently, this work is being undertaken in order to complete the genetic characterisation of BCoV field strains. Interestingly, one animal, which was febrile at the time of sampling, was positive for BCoV in nasal discharge as well as for BRSV in whole blood. It is likely that the subjacent BRSV infection had an impact on the immune status of the animal, favouring BCoV infection and excretion.

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THE FIRST AUSTRIAN CASE OF HEPATITIS CONTAGIOSA CANIS IN OVER 20 YEARS IN A DOG IMPORTED FROM HUNGARY

Viviane BENETKA[1], Ingrid GUTLEDERER[2], Muna LATIF[1], Karin WALK[1], Karin MÖSTL[1]

[1] *Clinical Virology, Department for Pathobiology, University of Veterinary Medicine, Vienna, Austria*
[2] *Small animal veterinary clinic Streit, Gerasdorf, Austria*

Keywords: Austria, CAV-1, HCC, hepatitis contagiosa canis, ICH

Introduction
Canine adenovirus 1 (CAV-1) is the causative agent of Hepatitis contagiosa canis (HCC; infectious canine hepatitis: ICH), a severe possibly fatal disease in dogs. After acute infection the virus, in many cases, establishes persistence in the renal tubular epithelium and is shed for an average of 6 to 9 months in the urine (Greene, 2006). The last report of 2 cases of HCC in Austria dates back to 1989 (Schwendenwein et al., 1989).

Case description
The case described here occurred in a juvenile (estimated age: 6 months) mixed breed with an unknown vaccination history originating from a Hungarian animal shelter. The dog had been imported to Austria by an animal welfare organisation. In the care of its new owner the animal developed severe disease symptoms: fever, dyspnoe, apathy and anorexia. A transient improvement after medical treatment was followed by blepharospasmus and unilateral uveitis ("blue eye"). Further clinical signs developed at the same time: gastroenteritis, polydipsia and polyuria. Retrospectively these might more likely be attributable to the severe pancreatitis and secondary diabetes diagnosed later. The clinical symptoms first raised suspicion for Canine distemper virus (CDV) infection. After Canine distemper was ruled out, HCC remained the most likely differential diagnosis, which was finally confirmed. To our knowledge this is the first case of HCC diagnosed in a period of more than 20 years in Austria. The dog recovered after several weeks including complete remission of the anterior uveitis.

Materials and methods
Conjunctival and pharyngeal swabs, EDTA-blood and urine were collected at the time of signs of acute disease and additionally urine samples repeatedly until 8 months after the onset of clinical disease. The samples served as templates for nucleic acid extraction (Viral RNA Kit, Qiagen, Hilden, Germany). For the detection of CAV-1/2 specific nucleic acids the following primers were used: forward primer: 5'-GCCACTACTCTCCTGTTGAT-3'; reverse primer: 5'-GAAGAAGAAGTCCGAGACAC-3' (Nell et al., 2000). The amplification product of 626 bp was sequenced (ABI PRISM, Genetic analyser) and submitted to a BLASTsearch in the GenBank database (www.ncbi.nlm.nih.gov/blast). For the detection of CDV specific nucleic acids the primers described by Frisk et al. (1999) were used.

Results
CAV specific nucleic acids were detected in the blood and the urine (Fig. 1), the PCR for CDV specific RNA was negative. The specificity of the PCR result was verified by sequence analysis, the detected sequence was to 100% identical to the CAV-1 GenBank reference (CAV-1 strain RI261, Acc. Nr. Y07760). Canine adenovirus specific DNA was detected in the urine for at least 8 months after the onset of clinical disease, although the animal had completely recovered (Fig. 2).

Conclusions and Discussion
An intensive trade of pet dogs exists between Hungary and Austria. Taking into account the high tenacity of CAV-1 and the extremely long shedding in the urine after infection, there is a highly underestimated risk of direct or indirect transmission of CAV-1 in particular in open access dog-runs in urban areas.

Hepatitis contagiosa canis was an "old" disease not seen in Austria for decades. We assume that this was not due to eradication of the causative agent but to high vaccination rates. In the light of the case described here, the importance of active immunization of an as high as reachable percentage of the dog populations cannot be emphasized enough.

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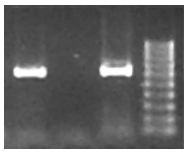

Figure 1: CAV-1/2 specific PCR
Lane 1 and 3: amplification product of the estimated length of 626 bp in blood and urine. Lane 4: molecular weight marker

Figure 2: After clinical recovery

MOLECULAR EPIDEMIOLOGY OF BOVINE CORONAVIRUS IN SWEDEN

Mehdi R. M. BIDOKHTI[1,2], Anna OHLSON[2], Madeleine TRÅVÉN[2], Stefan ALENIUS[2], Mikhayil HAKHVERDYAN[1], Sándor BELÁK[1]

[1] Joint Research & Development Division in Virology, Unit of Virology, Immunology & Parasitology (VIP), National Veterinary Institute (SVA), Uppsala, Sweden
[2] Division of Ruminant Medicine and Veterinary Epidemiology, Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural sciences (SLU), Uppsala, Sweden

Keywords: bovine coronavirus, phylogenetic tree, S1 subunit gene, semi-nested PCR

Introduction and objectives
Bovine coronavirus (BCV) occurs worldwide, causing infections associated with diarrhoea in newborn calves (CD), winter dysentery (WD) in adult dairy cattle and respiratory tract symptoms in calves and feedlot cattle (1,2,5). BCV belongs to the family Coronaviridae, order Nidovirales, and possesses a single-stranded, non-segmented, positive sense RNA genome of 32 kb plus poly (A) tail in length. The Spike (S) glycoprotein of the BCV facilitates viral attachment to susceptible cells. Its S1 subunit is associated with receptor binding functions (3). The purpose of this study was to investigate the molecular epidemiology of BCV in Swedish dairy herds based on the initial 1010 nt fragment of the S1 subunit gene.

Materials and methods
During 3 stable seasons, faecal samples were collected from calf diarrhoea (2005-2006) and winter dysentery outbreaks (2006-2008) in herds located in different regions of Sweden. A semi-nested PCR, based on the initial 624nt fragment of the S1 subunit, was optimized and used for virus detection (3,4). A second 386nt fragment of the S1 subunit of positive samples was also amplified using a nested PCR (3). Both fragments of PCR- positive samples were sequenced and aligned using BioEdit. Phylogenetic trees were constructed from the sequence alignment by using the neighbour joining-likelihood method of analysis of Mega 4.1 software. Reference strains of BCV retrieved from GenBank were used to assess the reliability of the method that was used for construction of the phylogenetic trees.

Results
Phylogenetically, most of the strains from the southern regions of Sweden and from the first two seasons clustered close to each other, while most of the strains from northern Sweden and the 3rd season clustered separately (Fig. 1). The sequence identity was 97-99% between southern strains and 96-100% between central and northern strains.

Discussion and conclusions
All the BCV strains from the 3rd season outbreaks belonged to two clusters of identical sequences. These two clusters showed 99.7% homology and may represent the circulation of a single strain in the northern and central parts of Sweden during season 3. The greater sequence variability among isolates from southern than from northern and central Sweden concurs with previous findings of a higher BCV prevalence in the southern parts of Sweden (6).

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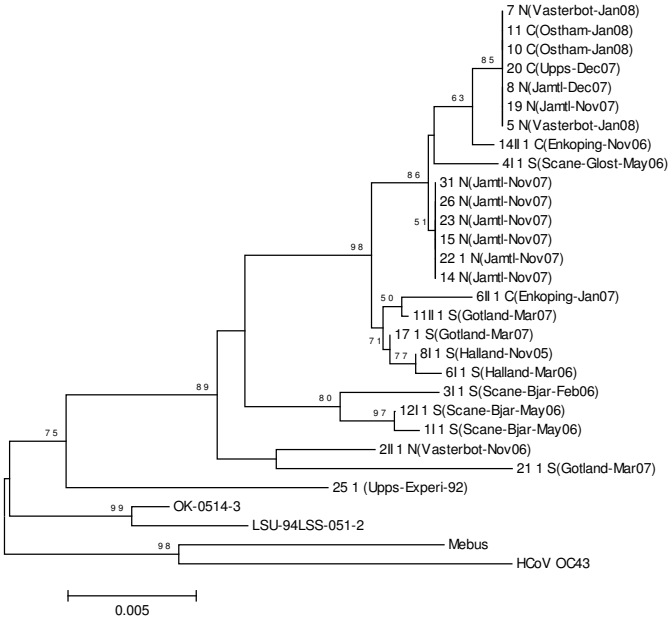

Fig. 1. Phylogenetic tree of BCV samples based on initial 1010 nucleotide sequence of S1 subunit gene. Capital letters represent the geographical region of the herds in Sweden; N for North, S for South, and C for Central.

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BoHV-4 INFECTION IN DAIRY CATTLE WITH REPRODUCTIVE DISORDERS IN TURKEY

Seval Bilge DAĞALP[1], Alireza Faraji MAJARASHIN[1], Tuba Çiğdem OĞUZOĞLU[1], Elvin ÇALIŞKAN[2], Başak ALTAŞ[3], Dilek MUZ[4], Feray ALKAN[1]

[1]Ankara University, Faculty of Veterinary Medicine, Virology Department, Ankara/TURKEY
[2]Central Veterinary Control and Research Institute, Etlik, Ankara-TURKEY
[3]Refik Saydam National Public Health Agency, Ankara-TURKEY
[4]Mustafa Kemal University, Faculty of Veterinary Medicine, Virology Department, Hatay/TURKEY

Key words: bovine herpes virus type 4, immunofluorescence, PCR, reproductive disorders, serology, virus isolation

Introduction and objectives

A number of viruses have been presumed to play role in reproductive disorders in cattle. Bovine herpesvirus type 4 (BoHV-4), is one of them, belongs to the family *Herpesviridae*, subfamily *Gammaherpesviridae* and species *Rhadinovirus*. BoHV-4 has no close biological and virological relationship to other known herpes viruses of the family bovidae (12). The persistent infection with BoHV-4 that was established in each species has been shown to interfere with the immune response (11). This effect of the virus may be a major contributory factor during both acute and latent BoHV-4 infection on the animals defence against other pathogens (7). The virus has been identified in the respiratory tracts of infected animals in addition to the cases of vulvovaginitis, endometritis, mastitis, abortion and also from apparently healthy cattle (1, 2, 5, 8, 10, 14). The presence of BoHV-4 infection in Turkey was reported by Bilge Dağalp et al.(3, 4).

In this study, it was planned that the prevalence of BoHV-4 infection was determined in cows with reproductive disorders in dairy herds in Turkey. In addition,the presence in leukocytes of BoHV-4 was investigated.

Materials and methods

Sampled animals A total of 1368 cattle aged ≥2 years old housed in 16 different dairy herds were investigated in this study (Herd no I-IV with reproductive disorders and X-XVI with healthy appearence). For this purpose 1368 sera samples, 120 vaginal swabs from animals with vaginal discharges and 8 aborted foetal samples were taken. In addition, 149 leukocyte samples from cattle with reproductive disorders were also tested for BoHV-4.

VI and IFA techniques All materials were passaged in Bovine Turbinatae cell cultures for three times and examined for the presence BoHV-4 virus by direct immunofluorescent technique. BoHV-4 reference strain used as control virus for PCR and immunofluorescent techniques was kindly provided by Dr.G.J.Wellenberg.

Polymerase chain reaction (PCR) technique DNA extractions from virological control samples were carried out according to Sambrook and others (13). The oligonucleotide primers specific for the BoHV-4 TK and gB region (7, 9) were used in the PCR amplification of the target fragments. The detection of BoHV-4 DNA was performed by use of the BoHV-4 PCR as described elsewhere by Wellenberg and others (15) with some modifications.

Indirect ELISA A commercial indirect ELISA (Bio X, Belgium, Bio K 066) used for the detection of antibodies against BoHV-4 was carried out according to manufacturer's recommendations.

Results

VI, and IFA techniques While BoHV-4 has been isolated from 14 (11.6%) vaginal discharge samples, 16.6% (20/120) of tested samples were found positive by IFA technique (Table 1, Figure 1A and B).The isolates were identified by PCR and IFA technique.

PCR technique Out of vaginal discharge samples 29.1% (35/120) were found to be positive for BoHV-4 by PCR (Table 1, Figure 2).

ELISA Out of 1368 blood sera tested 46.6% (637/1368) was found to be positive for BoHV-4 (Table 1). The serological data of cattle with and without reproductive disorders were compared. The seroprevalence rates of BoHV-4 infection were determined 52.3%(363/693) and 40.5%(274/675) in cattle with and without reproductive disorders, respectively .

Table 1: The results of virological and serological control according to sampled herds

Herd no	Vaginal swabs/abortus samples				Leukocytes		Serum samples	
	The number of samples*	PCR (%)	VI (%)	IFA (%)	The number of sample	PCR (%)	The number of serum	BoHV-4 Ab+ (%)
I	24/-	9(37.5)	3 (12.5)	4 (16.6)	36	16 (44.4)	176	88 (50)
II	1/2	1(100)/0	0	0	33	8 (24.2)	48	37 (77)
III	15/-	3 (20.0)	1 (6.6)	1 (6.6)	5	4 (80.0)	148	49 (33.1)
IV	7/-	3 (42.8)	0	0	21	7 (33.3)	96	21 (21.8)
V	1/-	0	0	0	-	-	23	11 (47.8)
VI	-	-	-	-	3	3 (100)	28	3 (10.7)
VII	13/3	3(23.07)/0	3/0 (23.07)	3/0 (23.07)	9	1 (11.1)	260	160 (61.5)
VIII	-/2	0	0	0	-	-	2	0 (0)
IX	55/-	16(29)	7(12.7)	12(21.8)	29	6 (20.6)	368	256 (*69.5*)
X	-	-	-	-	1	1 (100)	-	-
XI	-/1	1(100)	0	0	-	-	-	-
XII	-	-	-	-	-	-	79	0 (0)
XIII	4/-	0	0	0	1	1 (100)	45	1 (2.2)
XIV	-	-	-	-	11	-	50	11 (22)
XV	-	-	-	-	-	-	30	0 (0)
XVI	-	-	-	-	-	-	15	0 (0)
Total	**120/8**	**35/1 29.1 /12.5**	**14 (11.6)**	**20 (16.6)**	**149**	**47 (31.5)**	**1368**	**637 (46.5)**

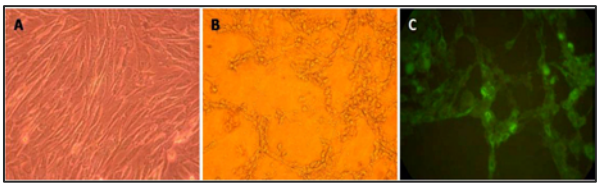

Figure 1. A. Cell control (Bovine Turbinata) X 400. B. The morphological changes of the field isolate K.339 in cell culture (2th passage) X 400 (4th day). C. The field isolate K.339 in immunofluorescent test X 800.

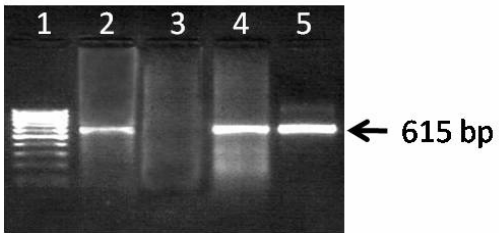

Figure 2. The agarose gel electrophoresis of PCR products Line 1, 100 bp DNA ladder (Fermentas, Lithuania); Lines 2 and 4, Positive samples; Line 3, Negative sample; Line 5, DN-599 (BHV4 reference strain)

Discussion and conclusions

In this study, the presence of BoHV-4 was investigated serologically and virologically in dairy cows with reproductive disorders receiving antibiotic treatment and animals without symptoms in the same dairy herds housed together during the sampling period and animals in different dairy herds with no reproductive problems.

The prevalence of BoHV-4 infection was found to be high in dairy herds with reproductive disorders. It is think that BoHV-4 may be an important factor in terms of the reproductive disorders in the sampled herds. Despite control programs against the BoHV-1 and BVDV infections (vaccination, eliminating persistently infected animals with BVDV, etc.) existence of remarkable reproductive disorders especially in herd I, III and IX may show the importance of the role of BoHV-4 infections on these cases. BoHV-4 persistence in leukocytes is reported in previous studies (6, 11), viral DNA determination in leukocyte samples provides an advantage for the evaluation of infection and give an idea about the persistence of the virus in leukocytes.

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FURTHER INDICATIONS TO THE VERTICAL TRANSMISSIBILITY OF GOOSE HAEMORRHAGIC POLYOMAVIRUS

Attila FARSANG[1], Sándor BERNÁTH[1], Mihály DOBOS-KOVÁCS[2]

[1]Central Agricultural Office, Directorate of Veterinary Medicinal Products, Budapest, Hungary;
[2]Faculty of Veterinary Science, Szent István University, Budapest, Hungary

Keywords: goose haemorrhagic polyomavirus, PCR

Introduction and objectives

Haemorrhagic nephritis and enteritis of geese (HNEG) is a fatal disease caused by goose haemorrhagic polyomavirus (GHPV). Since 1969, the first report of the disease by Bernath and Szalai (1970), the disease has spread through goose breeding areas of Germany (Schettler 1977, Miksch *et al*., 2002), Southern France (Schettler 1977, Guerin *et al*., 1999) and Hungary (Palya *et al*., 2004) with an epizootic pattern. The ways of viral transmission is a key issue for disease control measurements and HNEG epizootiology.

Sevaral observations posed the possibility of vertical transmissibility of goose polyomavirus including reported HNEG-related death in goslings at age of 4 days, contrary to the shortest incubation period of HNEG is six days. The presence of virus has not been confirmed in those cases. This study is the first report when GHPV presence was confirmed by pathological examination and LUX PCR in 4-day-old goslings showing typical symptoms of HNEG and originated from a field outbreak with well-documented disease history.

Materials and methods

Organ specimens

liver, intestine, kidney, lung, brain and Bursa Fabricii of three goslings died at age of 4 days (A, B and C) from a goose farm in Hortobágy region of Hungary, where 100 of 7033 recently hatched so-called Hortobágy White goslings died showing symptoms of nephritis and enteritis in 2008.

PCR

Real-time PCR assay applying light upon extension (LUX PCR) technique based on VP1 gene of GHPV. After PCR, melting curve analysis of the amplified PCR products was carried out from 48ºC to 96ºC in 0.5ºC/10 sec increasements and the melting curve was compared to a well-characterised positive control with known nucleotide sequence, which was amplified together with samples in the same assay.

Discussion and Results

The liver, spleen, kidney, intestine and Bursa Fabricii were PCR-positive for GHPV in case of gosling B and C, while only liver and intestine showed PCR positivity in case of goose A. GHPV was not detected in lung and brain specimen of any goslings. The findings of this study underline and clearly support vertical transmission of GHPV and are in concordance with our previous report when two experimentally infected geese embryos were able to hatch**.** The goslings showed poor physical conditions and died of HNEG at age of 4 days after hatching. GHPV was detected from cloacal swabs by PCR (Bernath *et al*., 2006). HNEG-related pathological lesions (Dobos-Kovacs *et al*., 2005) were manifested in birds including oedema in the subcutaneous connective tissue, mild nephrosis and haemorrhagic enteritis.

The vertically transmitted GHPV may infect goslings in the most susceptible life period, for this reason the vertical transmissibility of GHPV has profound significance on both disease control measurements and HNEG epizootiology.

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EFFECT OF PCV-2 ON THE SWINE TRANSCRIPTOME *IN VIVO*

Béatrice GRASLAND, Yannick BLANCHARD, Véronique BEVEN, Hélène FELIX, Anne-Cécile NIGNOL, Roland CARIOLET, André JESTIN

Agence Française de Sécurité Sanitaire des Aliments (Afssa),Unité de Génétique Virale et Biosécurité, 22440 Ploufragan, FRANCE

Keywords: circovirus, *in vivo*, microarray, transcriptome

Introduction and Objectives

Porcine circovirus of type 2 (PCV-2) is a small single-stranded circular DNA virus, associated with several diseases such as post-weaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS). Although PCV-2 is known as the aetiological agent of PMWS, co-factors are often required to trigger the disease (1). In order to determine which biological functions and which genes are involved in the PCV-2 pathogenicity, the transcriptome modifications were analyzed at early time of infection and at the time of the clinical expression in lymphoid tissue from piglets infected by PCV-2 and immuno-stimulated with a non-infectious PMWS co-factor.

Materials and methods

Five-week-old specific-pathogen-free (SPF) piglets were inoculated with 1ml intra-muscularly and with 5ml intra-tracheally of a PCV2 inoculum titrated at $10^{4.9}$ $TCID_{50}$/ml. The infected animals were also immuno-stimulated at 3 and 7 days post-infection (dpi) with 4ml of keyhole limpet hemocyanin emulsified in incomplete Freund's adjuvant injected by the intra-muscular route. Two control groups were included. The piglets of the first one were only immuno-stimulated and those of the second one received no treatment.

Piglets were necropsied at 3, 5, 10 and 23 days post-infection (dpi). Several lymphoid organs such as inguinal and tracheo-bronchial lymph nodes, and tonsils were collected, immediately frozen in liquid nitrogen and stored at – 80°C. Total RNAs were extracted from the tracho-bronchial lymph nodes and checked for integrity with Agilent CHIPS. The cDNA were synthesized from RNA, labelled and then hybridised on porcine microarrays spotted with 20 400 oligonucleotides corresponding to porcine genes. The normalized data were statistically analysed with the SAM software to assess the genes with variant expression. The main biological functions were determined by "Ingenuity pathway" analysis.

Results

The statistical analysis, performed with data obtained at 3 dpi, revealed 397 genes that showed variations in their expression, 343 of which were induced and 54 repressed. However expression variations were low and no main alterations of the transcriptome were identified. Only 2/9 genes with the highest and lowest variation in gene expression with micro-array data had their regulation confirmed by real-time RT-PCR. In the same way, no change of gene expression was detected at 5 and 10 days post-infection.

The microarray data obtained after 23 days post-infection, the time of PMWS clinical expression, were also statistically treated. At that time, the SAM analysis revealed 119 genes that showed variations in their expression. Five of these variants genes were induced and 114 repressed. The values of normalized log_2 signal ratio of the variant genes were higher than the ones obtained *in vitro* with minimum and maximum values of -1.4 and 1.08 respectively. Eleven down-regulated genes were selected for validation by real-time RT-PCR.

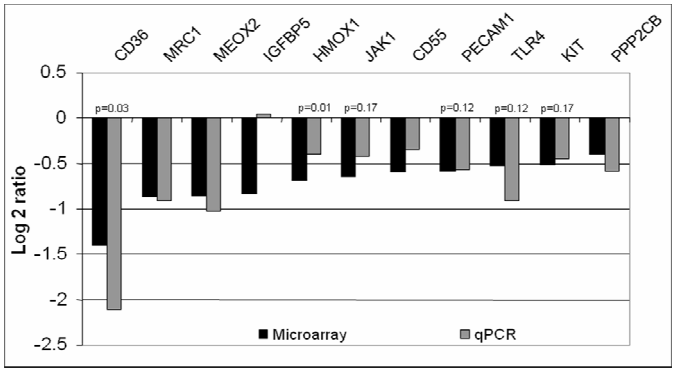

Figure 1: Real time RT-PCR results of 11 variant genes according to the microarray results at 23 day post-infection.

Among the 11 altered genes, two were significantly under-expressed by real-time PCR and four others tended to be significant (Figure 1). Thus the results of real-time PCR indicated that the microarray results are reliable. Ingenuity pathways analysis was performed to investigate function, biological processes and network interactions with the data obtained at 23 days post-infection.
Signal transduction, developmental process and response to stress were the major processes found (Table 1).

Table 1: Main biological processes characterized in PCV2-infected tracheo-bronchial lymph nodes at 23 dpi.

Biological process	GO Reference	Number of genes
Signal transduction	GO:0007165	26
Developmental process	GO:0032502	25
Response to stress	GO:0006950	10
Cell adhesion	GO:0007155	9
Vesicle-mediated transport	GO:0016192	8

Discussion and conclusions

Our results show that at the early time of PCV-2 infection, before the maximum viral load in the animal, the transcriptional response to PCV-2 infection remains low. Moreover, it seems that the modifications in gene expression caused by the viral infection at 5 and 10 dpi were hidden by the immuno-stimulation used in the experiment.

On the contrary, at 23 dpi, the host reacts to the viral infection by repressing the expression of genes mainly involved in the signal transduction as well as in the response to stress including the immune response.

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PHYLOGENETIC AND ANTIGENIC CHARACTERISATION OF CHICKEN ASTROVIRUSES - IDENTIFICATION OF A NEW, GENETICALLY AND SEROLOGICALLY DISTINCT GROUP

Tamás MATÓ, Edit FODOR, Tímea TATÁR-KIS, Vilmos PALYA

CEVA-Phylaxia Biologicals Co. Ltd., Szállás u. 5. H-1107 Budapest

Keywords: chicken astrovirus, cross-neutralisation, PCR, phylogenetic analysis

Introduction and objectives

Astroviruses are nonenveloped, positive-sense RNA viruses, 28-30 nm in diameter with star-like morphology. Their genome is 6.8-7.9 Kb in length, has three open reading frames, 5' and 3' untranslated region and poly-A tail. ORF1a encodes the non-structural proteins, ORF1b encodes RNA-dependent RNA polymerase and ORF2 encodes the capsid proteins. There is a retrovirus-like frameshift structure between ORF1a and ORF1b and the capsid proteins are expressed as one long precursor protein from a subgenomic RNA (3).

In poultry, Astroviruses have been associated with enteritis, uneven growth and increased mortality in young turkeys and chickens, nephritis in chickens and a fatal hepatitis in ducklings. Serologically and genetically the chicken-origin astroviruses were divided into two groups: chicken astrovirus 1 (CAstV1), also called avian nephritis viruses (ANV) (2) and recently identified chicken astrovirus 2 (CAstV2) (1, 4) Due to the difficulties to grow astroviruses in vitro, the most useful diagnostic tool for the detection of the causative agent is the RT-PCR.

The objective of this study was to examine the genetic diversity of chicken-origin astroviruses isolated in Hungary and to compare them with isolates from other countries.

Materials and methods

Between 1999-2008 twenty four different Hungarian flocks exhibiting uneven growth and elevated mortality due to nephritis and uricosis or enteric disorders during the first 2-3 weeks of life were investigated for astroviruses by RT-PCR. Four Polish, one Turkish, two Peruvian and two Columbian avian astrovirus isolates were also included in the study. Two sets of primer pairs covering the most conserved region of the polymerase gene (ORF1b) were designed for the detection of astroviruses. The first set of primers amplified a 691 bp PCR product spanning from 3544 to 4235 nt and the second set of primers amplified a 528 bp PCR product between 3607 and 4135 nt. The PCR products were sequenced and the sequence differences were calculated by the Kimura's method and phylogenetic tree, including available sequences from the GenBank, was constructed using the Neighbour-joining method by TREECON for Windows program.

For virus isolation tissue suspensions of kidney and small intestine from affected chicks were inoculated into embryonated chicken eggs via the yolk sac route. Allantoic fluid and internal organs of the embryos showing gross pathological lesions indicative to astrovirus infection were harvested. The homogenized embryo and allantoic fluid was used for the inoculation of LMH cell culture. After 2-3 days of incubation, cytopathic effect of the virus could be observed. Titre of the virus suspensions was determined using standard micro-neutralization method on LMH cells. Titre was calculated according to the method of Spearman-Kärber.

Eight isolates belonging to genetically different groups of chicken astroviruses and the referent ANV strain (Weybridge) have been chosen for serological characterization. Hyperimmune serum was produced in SPF chickens kept in negative pressure isolator units. Chickens were immunized first by live virus suspension administered per os and then boosted with inactivated, oil adjuvanted antigen prepared from the same virus suspension. Serological relationships of the virus strains were tested by cross-neutralization method and by indirect immune-peroxidase staining using the relevant hyperimmune serums. Titre of the hyperimmune serum samples was determined against homologous and heterologous virus strains. Virusneutralization was performed according to standard ß-method in LMH cell culture against 50-100 $TCID_{50}$ virus. Virusneutralizing titre of the serum samples was calculated as the theoretical serum dilution which can protect 50% of the cells from the cytopathic effect of the virus. Ratio of protected cells was determined on the basis of absorbance values measured after staining with the MTT (Thiazolyl blue) vital dye. Serological relationship between two viruses was described by the value of relatedness (r), calculated according to the *Archetti és Horsfall* formula (1). Virus strains exhibiting r values greater than 0.7 belongs to the same serotype, strains showing r values in the range of 0.5-0.7 are different subtypes within the same serotype. R value smaller than 0.5 is indicative of different serotype. Immune peroxidase staining was performed on chicken astrovirus infected LMH cell cultures showing ~40% CPE, using standard method. Each virus strain was tested against homologous and heterologous serum, results were evaluated as positive (binding of antibodies to the infected cells) or negative (no viral proteins detected).

Results

Fourteen of the twenty four Hungarian flocks, all Polish, the Peruvian, the Turkish and the two Columbian isolates resulted in positive PCR product by one of the two primer sets. The first primer set was able to detect those isolates that clustered into the CAstV1 (ANV) group and the ones which related to CAstV2 group published in the literature. From some of the samples, however, astrovirus RNA could be amplified only with the second set of primers. These isolates formed a new separate group of chicken astroviruses called CAstV3, which is phylogenetically more closely related to CAstV2 group than to ANVs. The majority of isolates fell into CAstV2 or CAstV3 groups and only five of the Hungarian isolates grouped together with the avian nephritis viruses (CAstV1). Two Columbian, two Peruvian, the Turkish and four Hungarian isolates grouped together with CAstV2 strains. The four Polish and five Hungarian isolates form the new separate group: CAstV3. The largest divergence within the ANV (CAstV1) group was 13%, in the "CAstV2" group was 16% and in the "CAstV3" group was 8%. The biggest distance between the CAstV2 and CAstV3 groups was 27%. Sequence difference between the ANV and CAstV2-3 groups was significantly larger, showing 49% distance between the two groups.

Five serotypes could be differentiated on the basis of cross-neutralization test. ANV reference strain belonged to a separate serotype. Both strains tested from CAstV3 group belonged to another serotype. There was a great heterogeneity in the CAstV2 group, as 3 serotypes could be identified among the four strains tested. One isolate in the CAstV1 group was characterized as a subtype of one CAstV2 serotypes. Peroxidase-staining based characterisation verified the unique serologic properties of ANV reference strain. Both CAstV3 strains tested belonged to another group along with the majority of CAstV2 strains. One CAstV2 isolate belonged to a third, separate group. The isolate tested in CAstV1 group reacted with all hyperimmune sera (including anti-ANV), indicating the presence of a common antigen(s) in this strain with all other serological groups.

Discussion and conclusions

The results of the phylogenetic analysis showed that astrovirus isolates from chickens clustered into three separate groups including ANV group (CAstV1) and two groups of the so-called chicken astroviruses: CAstV2 and the newly identified group, CAstV3.

It can be concluded that the genetic divergences among poultry astroviruses are so large, even in the most conserved region of the genome, that several primer sets should be used to reveal new groups of CAstVs.

Cross-neutralization test verified the great heterogeneity among CAstV strains, as 5 different serotypes could be identified among the 8 strains tested. Immune-peroxidase staining differentiated 3 antigenic groups among the strains: (i) CAstV1; (ii) CAstV3 and most CAstV2; (iii) and one strain in CAstV2. One isolate tested seems to be a recombinant strain belonging to CAstV1 on the basis of polymerase gene sequence, closely related to a CAstV2 strain on the basis of cross-neutralization and sharing antigens with all CAstV genetic groups as detected by the immune-peroxidase method.

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THE GENOMIC CONSTELLATION OF A NOVEL AVIAN ORTHOREOVIRUS STRAIN ASSOCIATED WITH RUNTING STUNTING SYNDROME

Krisztián BÁNYAI[1, 2], John K. ROSENBERGER[3], Tamás MATÓ[4], Vilmos PALYA[4]

[1] *Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary;*
[2] *Department of Medical Microbiology and Immunology, Faculty of Medicine, University of Pécs, Pécs, Hungary;*
[3] *AviServe LLC, Newark, DE, USA;*
[4] *CEVA-Phylaxia Veterinary Biologicals Co. Ltd, Budapest, Hungary*

Keywords: chicken, phylogenetic analysis, reovirus, RSS

Introduction and objectives

Avian orthoreovirus is a species within the genus *Orthoreovirus*, family *Reoviridae* and is represented by numerous strains from domesticated and wild bird species (1).

Avian orthoreoviruses (ARVs) account for significant losses in commercial poultry. In chicken, ARVs have been associated with tenosynovitis, chronic respiratory disease, and malabsorption syndrome.

Despite the economic importance, molecular characterization of reference strains is limited to a subset of the genomic segments, mainly to the S-class genes. Strikingly few sequence data are available for the M- and L-class genes. Of interest, neither fully sequenced avian orthoreovirus genome nor the full genomic constellation based on partial genome segments is available for any particular ARV isolates. Despite these shortcomings, the genome size (~23.5 kbp) can be predicted on the basis of existing sequence information.

ARV strains have been associated in chicken with runting stunting syndrome (RSS) for several decades. One such strain was selected for full genome sequencing. We present here the molecular characterization of the full-length genome sequence of the ARV strain, D999/2.

Materials and methods

Virus strain. The strain D999/2 was isolated from 12 days old broiler chick suffering from RSS in the USA. It was isolated and serially passaged on LMH cells. At the 5th passage the virus was harvested and then subjected to genome sequencing.

Laboratory methods. The genomic RNA was extracted by the TRIzol method (2). Preliminary sequence information was obtained by gene specific RT-PCR amplification of fragments of each genome segment. A short oligonucleotide was ligated to the 3' ends of the genomic RNA, and then its complementary primers were used in combination with gene specific primers to determine the 5' and 3' ends of the genomic segments. Nucleotide sequences were determined by the dideoxy chain termination method.

Computer analysis. Contigs were constructed and edited using the GeneDoc software (4). Phylogenetic analysis was carried out using the MEGA4 software (neigbor-joining algorithm, *p*-distance model, bootstrap analysis) (5).

Results

The full-length genome (23,494 bp) of strain D999/2 was determined by the primer walking sequencing strategy. The length of untranslated regions ranged 12 nt to 30 nt at the 5' end, and 30 nt to 98 nt at the 3' end. The most 5' and 3' end sequences were conserved in all genome segments (5' end, GCUUUU(U). 3' end, UCAUC).

The L genome segments. The L1 genome segment of D999/2 was most closely related to strain 918 (91% identity), while the L3 genome segment shared the highest nt identity with strains 1017-1 and R2 (89%). Only 2 published L2 genome segment of chicken ARV have been published so far (strains 138 and 176); our strain was more closely related to strain 138 (91% nt identity)

The M genome segments. Comparison of the M1, M2, and M3 genome segments revealed moderate to high sequence identities to other ARV strains, being the most closely related isolates 138 (M1, 90% identity), 601G (M2, 85% identity) and OS161 (M3, 88% identity).

The S genome segments. The S1 genome segment was found to be tricistronic with partially overlapping open reading frames (ORFs); the position of the 3 ORFs (p10, p17 and σC) was similar to that of seen in other chicken ARV strains. The σC coding region shared low nt identity to the majority of strains (30%-70%), except for GA 40973/2005, (93%) isolated from an idependent case of RSS in the USA. With regard to the S2, S3 and S4 genome segments, D999/2 shared the highest nt identity to 138 (S2, 93%; S3, 92%), and to recent US chicken ARV strains (up to 93%).

Discussion and conclusions

While the availability of limited sequence information allowed gaining insight into the overall structure of ARV strains, this study is the first to report on a full-length ARV genome sequence. The length of the genome fell in the size range predicted based on full-length cognate genome segment sequences available for a handful of reference ARV strains.

The majority of genes of D999/2 shared significant degrees of sequence conservation with related genes of prototype ARV strains (≥85%). However, the S1 segment of the genome represented an exception. This genome segment shared only ≤67% nt identity with corresponding genome segment of other ARV strains. On the other hand, the σC protein encoding region of the S1 genome segment was closely related to another US ARV strain isolated also from RSS.

The low number of ARV gene sequences and the lack of absolute sequence identities with related strains did not permit us to identify the possible parental strains of our novel strain. Nonetheless, we had convincing evidence that accumulation of point mutations and reassortment of cognate genome segments played key role in the origin and evolution of the strain D999/2.

Although a previous study did not identify a correlation between disease manifestation and genotype specificity of the σC (the outer capsid protein responsible for cell attachment and thus likely involved in tissue tropism) (3) the recent identification of strains carrying a novel σC protein from cases of RSS by independent research groups seems to be of interest and warrants further studies to be conducted.

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GENETIC CHARACTERIZATION OF CANINE DISTEMPER VIRUS (CDV) FROM DOGS AND RED FOXES IN GREECE

Charalambos BILLINIS[1, 2]

[1] *Laboratory of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Thessaly, 224 Trikalon, 43100 Karditsa, Greece;*
[2] *Institute of Biomedical Research and Technology, 51Papanastasiou, 41222 Larissa, Greece.*

Keywords: canine distember virus, dogs, phylogenetic analysis, red foxes

Introduction and objectives

Canine distemper virus (CDV), a member of the genus *Morbillivirus* in the *Paramyxoviridae* family, is the causative agent of a systemic, often lethal, disease of domestic and wild *Canidae*. CDV disease in wild and captive foxes are numerus and distemper-like neurological signs and histological lesions in the grey fox have been described. Serological investigations for CDV in the fox populations have revealed a prevalence of about 4-11% (1). These data suggest that the fox may also be a natural host for CDV and that infected dogs might serve as sources of virus for wildlife species or vice versa. The genome of CDV encodes the following proteins: matrix (M), fusion (F), hemagglutinin (H), nucleocapsid (N), polymerase (P) and phospoprotein (P). The H protein is responsible for viral attachment to the cell host and may also play a role in induction of protective immunity. The highest genetic variability has been found in the H protein gene and so this is the most appropriate protein to be monitored for detection of genetic changes of the virus (2). In this study, phylogenetic characterization on complete H gene from twelve isolates from dogs and three CDV isolates from foxes in Greece was performed. The extent of genetic variation among these isolates and all other known CDV isolates was analyzed.

Materials and methods

Cases

Twelve dogs, manifesting central nervous signs and so suspect of CDV infection were included in this study. Eight dogs were males and four females. Six were purebreds (dogs Nos 1, 4, 5, 8, 9 and 10) and six were crossbreds (dogs Nos 2, 3, 6, 7, 11 and 12). The age of these animals ranged from 4 months to 5 years. Four dogs (Nos 1, 4, 8 and 9) had been vaccinated against canine distemper. After euthanasia, CNS tissue samples were collected for RT-PCR. In addition, twelve red foxes were found dead. Tissue samples of spleen and lung were submitted to our laboratory.

RNA extraction, PCR amplification and sequence analysis

RNA was isolated using a RNA isolation kit (Gentra Systems, Minneapolis USA) according to the manufacturer's instruction. Reverse transcription and PCR conditions were the same as described previously (4). Sequence analysis was performed commercially by MWG Biotech (Germany). Nucleotide sequences from the other CDV isolates were retrieved from the EMBL database. Phylogenetic and molecular evolutionary analyses were conducted using program MEGA 3.1.

Results

Extracted RNA from CNS tissue samples of all twelve dogs was positive for CDV. A different virus strain was isolated from each dog. We designated each isolate by the godes Dogs GRE 1 to 12. Extracted RNA from pooled tissue samples from three foxes was positive for CDV. A different virus strain was isolated from each fox. We designated each isolate by the godes Fox GRE 1 to 3. Sequence analysis showed that the complete haemagglutinin (H) gene was 1824 bp in length in all fifteen Greek canine distemper viruses. Phylogenetic analysis was performed on the complete H gene of 61 CDV isolates, including the fifteen Greek isolates described in this study. This analysis revealed that the homology of the nucleotide and amino acid sequences between the twelve Greek isolates from dogs was 96.9-99.2% and 95.8-99.1%, respectively. In addition, the homology of the nucleotide and amino acid sequences between the three Greek isolates from foxes was 97.1-99.4% and 95.9-98.5%, respectively. The homology of the nucleotide and amino acid sequences between the Greek isolates and the vaccine strains was 91.3-92.1% and 90.3-91.4%, respectively. Eleven Greek CDV isolates from dogs and two Greek CDV isolates from foxes clustered together with the recent European strains from dogs. The twelfth isolate from dog, along with a Greek fox isolate, a ferret strain from Germany and a mink strain from Denmark were more distantly related to the major European lineage (Fig. 1).

Discussion and conclusions

The detection in red foxes CDV isolates similar to CDVs from dogs might indicate that interspecies circulation between dogs and wild carnivores may occur. The possibility that, under particular conditions, wildlife species might act as a reservoir of CDV infection for domestic dogs or vice versa, should be better addressed by analysis of additional CDVs from wildlife animals (3). Recently, the number of dogs diagnosed as canine distemper has increased all over the world. Many of these affected dogs had vaccination histories (5). These facts suggest the appearance of CDVs with antigenicities different from those of the vaccine strains. Two Greek CDV isolates from foxes fell on single lineage which included the eleven Greek and all the other European strains from dogs and are clearly separated from vaccine strains as well as strains from Japan, Asia and USA. The third fox CDV isolate along with the twelfth Greek isolate from dog were distantly related to the CDV isolates of the European lineage. Due to the few epidemiological surveys and to different genes targeted in the various studies, the distribution of the major CDV lineages throughout the world is not clear. Analysis of CDV isolates detected globally and from a variety of host species will provide a more understanding of the ecology of virus and will provide the basis for improvement of current CDV vaccines.

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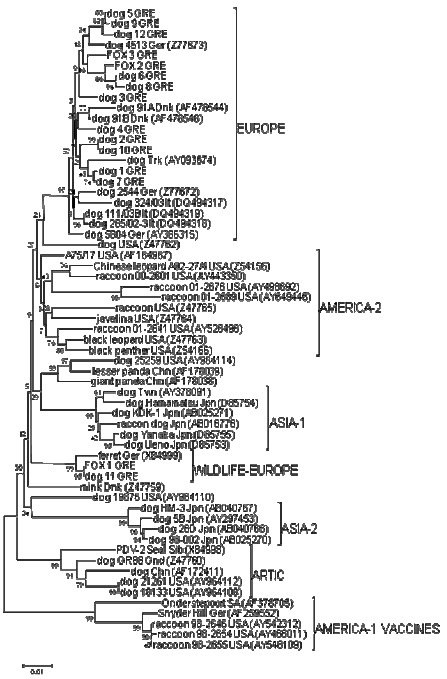

Figure1. Neighbor-joining tree showing the relationship of CDV isolates

ENTIRE GENOMIC CHARACTERISATION OF 10 LOW PATHOGENIC H5 SUBTYPE AVIAN INFLUENZA VIRUSES COLLECTED IN FRANCE BETWEEN 2002 AND 2008

François-Xavier BRIAND, Ghislaine LE GALL-RECULÉ, Katell OGOR, Cécile GUILLOU-CLOAREC, Aurélie BLUTEAU, Céline MACÉ, Séverine FERRÉ, Audrey SCHMITZ, Pascale MASSIN, Olivier GUIONIE, Josiane LAMANDÉ, Chantal ALLÉE, Martine CHERBONNEL, Jean-Paul PICAULT, Véronique JESTIN

AFSSA, Avian and Rabbit Virology, Immunology and Parasitology Unit (VIPAC), French Reference Laboratory for Avian Influenza and Newcastle Disease, B.P. 53, 22440 Ploufragan, France

Avian Influenza, low pathogenic H5 subtype, genotyping.

Introduction and objective

Aquatic birds are the primary reservoir of type A Influenza viruses. These avian influenza viruses (AIV) are classified into subtypes based on antigenic differences in their surface glycoproteins (hemagglutinin and neuraminidase). The AIV genome is segmented into eight negative strand allowing reassortment events. The genetic evolution of these viruses can have different origins including mutations, insertions, deletions and reassortment. Indeed, when at least two viruses infect the same cell of a given host, the viruses resulting from this coinfection can mix segments from either parental viruses, such a phenomenon having given rise to pandemic viruses. Moreover, infection of poultry with H5 or H7 LPAI viruses can either be asymptomatic or cause a wide range of signs varying from mild respiratory disease to more severe diseases in case of aggravating circumstances. Moreover these viruses could become highly pathogenic. The monitoring and the characterization of H5 and H7 AIV LP from wild birds and domestic birds is essential to improve the knowledge of AIV genetic evolution and to further understand the consequences of new mutations. We presently analyzed 10 whole genomes of AIV LP of H5 subtype isolated from decoy ducks (2), commercial ducks (7) and chickens (1) during 6 years, between 2002 and 2008 in France. Our purpose was i) to determine the relationships of each RNA segment with its counterpart from previous eurasian isolates ii) to assess the range of diversity of genotypes, iii) to determine whether specific combinations of genes might be favoured, iv) to identify particular molecular traits through each segment.

Materials and methods

The H5 LP avian viruses mentioned above belonged to our collection as a reference laboratory and split into 2 H5N1, 3 H5N2 and 5 H5N3. Genomic influenza RNA was extracted from 200µL of allantoic fluid harvest using QIAamp viral RNeasy Mini kit as described in the kit protocol. RNA was reverse transcribed by using Superscript II, hexanucleotides or a specific primer of all influenza segments. cDNA was completely amplified by gene specific primers for each of 8 genes using Platinum Taq DNA polymerase For PB2, PB1 and PA, the full genes were obtained following 2 overlapping PCR. The other primers were defined in laboratory (primers available upon request). A phylogeny, was inferred using MEGA3.1 with the Neighbor-Joining analyses and confirmed by PhyML for the Maximum Likelihood analyses. About 250 Eurasian lineage complete avian sequences were selected for each influenza segment. These sequences were obtained from representative of different subtypes and locations that were available in databases. The next step was to determine which genetic group was related each of French H5 LP genes with. For that, 12 Eurasian strains, completely sequenced, that shared at less 2 genes belonging to the same subgroup as French viruses, were selected as prototype viruses. Subgroups were respectively determined for each of the 8 genes. The subgroups were validated by bootstrap value superior to 75% and confirmed by the P-values test ($p>0.01$) with PhyML. For each identified subgroup, at least one or more French viruses and prototype viruses were present. If several prototype viruses clustered in the same subgroup, then the reference virus was the oldest selected viruses.

Result

The result of the phylogenic study showed that for each other gene of AIV FP of H5 subtype isolated in France, at least 2 genetic groups were identified. All the studied genes belong to the Eurasian avian lineage of the influenzavirus. We also showed that French LP AIV had a common origin with Italian AIV, Dutch, Danish like some Asian viruses. However, the distribution of the sequences in the different genetic groups composing the trees produced for each of 8 genes was very different. Thus, the schematic representation of the different viruses according to their genotype shows that out of the 10 French viruses, only 3 H5N3 viruses (isolated within a 4 months interval) had the same genotype. Many original characteristics were found on the genes PB1, NA, M2 and NS. Thus, the addition of a glutamine in C-terminal position was identified in 3 out of the 10 studied PB1. It was also noted that protein PB1-F2 of the chicken virus was truncated (11 amino-acids (aa) versus 78 aa normally. The N2 neuraminidase the chicken virus presented a deletion of 19 aa in its N-terminal as a trait of its adaptation to chicken. Concerning the M2 segment, a mutation (I28V) was observed for one H5N1 from duck. Another characteristic was also highlighted on this virus: on NS1 protein had an addition of 8 aa in C-terminal position. Finally, we observed the mutation A42S on the gene NS1 of 6/6 strains having allele A.

Discussion and conclusion

The sequencing of 10 complete genomes of AIV LP H5 subtype isolated in France between 2002 and 2008 allowed to characterize these viruses genetically and to highlight reassortant events. This study showed the existence of many genotypes among French H5 LP viruses. In addition, the 10 French LP H5 viruses isolated from birds in contact with the wild birds as well as the virus isolated from chickens reared without good hygiene, had genes of common origin with those characterized in wild species. These results confirm the existence of multiple co-circulations and viral co-infections within the populations of birds, thus increasing the probability of appearance of potentially pathogenic AIV for poultry. It would be important to study the implication of the molecular characteristics described in this study on French AIV, in particular in terms of pathogenicity and contagiousness.This work is scheduled in our laboratory while resorting to reverse genetics and the possibility of in vivo experimentations in high containment facilities.

ADAPTATION OF AVIAN INFLUENZA VIRUSES (SUBTYPE H9) TO DIFFERENT AVIAN HOSTS

Meike DIEDERICHS[1], Maren BOHM[1], Henning PETERSEN[2], Christine WINTER[1+2], Silke RAUTENSCHLEIN[2], Christel SCHWEGMANN-WESSELS[1], Georg HERRLER[1]

[1]Institute of Virology, University of Veterinary Medicine, Hannover, Germany;
[2]Clinic for Poultry, University of Veterinary Medicine, Hannover, Germany

Keywords: adaptation, avian influenza, hemagglutinin, sialic acids, tracheal organ cultures

Introduction and objectives

Influenza viruses initiate infection of their host by binding of the haemagglutinin (HA) to sialic acid residues presented by cell surface components. Viruses isolated from different host species may differ in their preference for the type of sialic acid, e.g. N-acetylneuraminic acid (Neu5Ac), or for a linkage type connecting the sialic acid molecule to the neighbouring sugar. Influenza viruses from mammalian hosts have a preference for α-2,6-linked sialic acids (1). Most avian influenza viruses prefentially recognize Neu5Ac attached via an α-2,3-linkage to galactose. An exception are avian viruses of the H9 subtype. Several strains of H9 influenza viruses have been shown to have a preference for α-2,6-linked sialic acids (2). This finding was surprising because in some avian species α-2,6-linked sialic acids are expressed in very low amounts, at least in the respiratory epithelium, a primary target for influenza virus infection.

Studies with tracheal organ cultures (TOC) have revealed that infection of the chicken tracheal epithelium by H9N2 influenza virus is not sensitive to pretreatment with neuraminidase. This may be related to results obtained by lectin staining indicating that the surface of the tracheal epithelium contains α-2,3-linked but no detectable amounts of α-2,6-linked sialic acid (Bohm et al., in preparation). The virus used for these experiments has been propagated in embryonated chicken eggs. We wondered whether adaptation of the virus to the tracheal epithelium after several infection cycles in TOCs would improve the replication efficiency and cause a change in the binding properties of the virus.

Materials and methods

Influenza virus of the N9N2 subtype was used to infect tracheal organ cultures (TOC). TOCs were prepared by manual cutting of the trachea of 20 days old chick embryos into rings about 1mm thick. For comparison, TOCs from turkey embryos (25 days old) were used. The rings were cultured in an over-head shaker.

The epithelial cells of TOCs were viable for up to 14 days as indicated by the ciliary activity of the epithelium. Infection by avian influenza virus induced ciliostasis which was used for monitoring the infection.

Results

To analyze the adaptation process of H9N2 virus in the tracheal epithelium, egg-grown stock virus was passaged four times on either chicken or turkey TOCs. The respective viruses were subjected to an analysis with respect to their replication behaviour as well as to their binding preferences.

The adaptation process was evident when the ciliostasis of the virus was determined. Compared to the original virus, the adapted virus induced a comparable ciliostatic effect in a time that was one day shorter. Also the infection characteristics were different. Whereas the starting virus was resistant to pretreatment of chicken TOCs with neuraminidase, the adapted virus showed some sensitivity to enzyme pretretment, though it was less sensitive to neuraminidase pretreatment than was the infection of turkey TOC. How these differences are reflected in the recognition of sialic acids, is currently under investigation.

To determine the molecular basis of these differences, we have started to determine the sequence of heamagglutinin proteins of the original virus and the adapted virus. Results of this analysis will be presented.

Discussion and conclusions

The binding properties of influenza viruses are often described in an oversimplified way saying that mammalian influenza viruses recognize α-2,6-linked sialic acids and avian influenza viruses α-2,3-linked sialic acids. Apart from the fact that influenza viruses usually have a preference for rather than an exclusive recognition of a certain linkage type, viruses of the H9 subtype clearly demonstrate that there are also avian influenza viruses with a preference for α-2,6-linked sialic acids. In a parallel contribution we have demonstrated that the same virus may use different receptors for infection of chicken and turkey TOCs (Bohm et al.).

Our data extend these findings showing that the optimal growth conditions may be different in eggs, chicken TOCs or turkey TOCs. The adaptation process to these optimal growth conditions is at least in part related to the interaction with receptors, because the virus that had been passaged four times on chicken TOCs showed some sensitivity to neuraminidase whereas the egg-grown H9N2 virus was resistant to neuraminidse pretreatment of chicken TOCs.

It will be interesting to see what amino acid changes in the haemagglutinin are involved in the adaptation process and how they affect the recognition of sialic acid.

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GENOME ANALYSIS OF A FISH ADENOVIRUS CONFIRMS THE PROPOSAL FOR A FIFTH ADENOVIRUS GENUS

Andor DOSZPOLY, Balázs HARRACH, Mária BENKŐ

Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary

Keywords: adenovirus, genome analyses

Introduction and Objectives

Adenovirus-like particles have been observed in a number of different fish species including cod, dab, Japanese eel and sea bream but to date, only one single isolate, from white sturgeon (Acipenser transmontanus), is available. Initial genetic study of this adenovirus (WSAdV-1) indicated its considerable phylogenetic distance from members of the four established adenovirus genera(1, 2). Thus the hypothesis on the co-evolution of AdVs with their respective vertebrate hosts seemed to be supported (3). Sequencing of the central genome part, between the genes of the DNA polymerase and the 33 K protein, has revealed an organisation conforming to the conserved core region of every adenovirus. In the present work, analysis of the proximal regions, possessing several astonishing features, is presented. Although the amount of genomic sequence, determined from WSAdV-1 to date, exceeds 30 kb the ITRs could not be identified yet.

Materials and methods

The nucleotide (nt) sequence of the central genome part of WSAdV-1 has been determined with the combination of molecular cloning and joining the clones by PCR. However a large cloned viral DNA fragment, obviously coding for fibre sequences, could not be properly merged with the supposedly closest neighbouring sequence of the gene of the 33 K protein. To obtain the genome ends of WSAdV-1, unidirectional PCRs with long (>26 nt) primers was attempted on both sides of the assembled contig. In several instances, instead of the expected single stranded PCR products, short double stranded fragments were generated with the same primer attaching to both ends. The nt sequence of such a product revealed fibre like motifs thus indicating the correct position of the cloned fibre region. A "bridging" PCR indeed confirmed the location of two fibre like genes on the left side of the genome. On the right hand side, several novel ORFs with unknown functions were recognised.

Results

Presently, a more than 30 kb long continuous sequence, with an average G+C content of 44%, is assembled as shown below. Interestingly, left from the gene of IVa2, more than 6200 bp sequence was determined with at least four putative genes. Two of them, in rightward orientation, seem to code for fibre proteins. The deduced protein product of the third full gene shows closest similarity to different bacteriophage proteins, whereas the putative product of the leftmost located partial ORF resemles bacterial proteins. On the right end of the contig downstream from the 33K gene, more than 4000 bp sequence was determined. Several, putative small ORFs were recognized here, but none of them show clear homology to any proteins known to date. No homologues of the pVIII or U exon were found either. It is noteworthy, that the homologue of pVII of WSAdV-1 is very short possessing a single protein cleavage site compared to two such sites in every other AdV studied before.

Discussion and conclusions

Our results modify the comprehension about the gene set initially thought to be fully conserved by every member of the family Adenovirdae in as much as the genes of pVIII and the U exon proved not to be constituents of this set. Moreover, the location of the fibre gene is also different than that in the previously studied adenoviral genomes. To date, siadenoviruses, having the shortest adenoviral DNA with no more than 5 genes or ORFs in addition to the conserved core, had been considered as the most ancient type of AdVs (4). Since one of the merely two siadnoviruses was isolated from frog, this lineage had tentatively been assumed to represent AdVs co-evolving with the amphibians. Genome analysis of the fish AdV strongly challenges this hypothesis. Moreover, in the past couple of years, several novel siadenovirus types have been detected in different bird species. Therefore it is likely that the real amphibian AdVs are yet to be discovered. Completion of the genome sequencing of WSAdV-1 might facilitate targeted screening for newer fish and perhaps amphibian AdVs.

Acknowledgements

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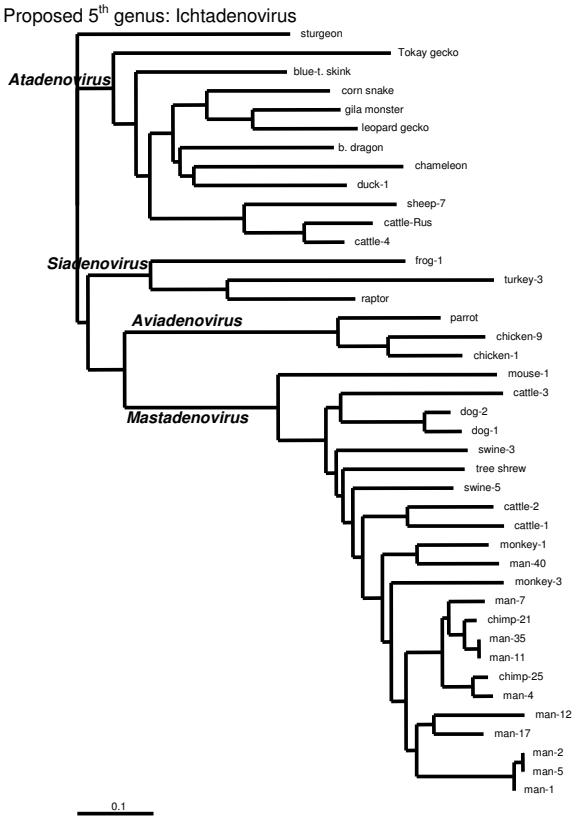

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PRELIMINARY STUDIES IN THE DEVELOPMENT OF A MINOR-GROOVE BINDER PCR ASSAY FOR THE PHYLOGENETIC CHARACTERISATION OF PCV2 ISOLATES

Catherine Duffy[1], Julie McClintock[2], Michael McMenamy[1], John McKillen[2], Prof. Gordon M. Allan[2]

[1]Virology Branch, Veterinary Sciences Division, Queen's University Belfast, Stoney Road, Stormont, Belfast, Co. Antrim, Northern Ireland, BT4 3SD;
[2]Virology Branch, Veterinary Sciences Division, AFBI, Stoney Road, Stormont, Belfast, Co. Antrim, Northern Ireland, BT4 3SD.

Keywords: MGB, PCR, PCV2, genogroup

Introduction and Objectives

Porcine circovirus type 2 (PCV2) is a small, non-enveloped, single-stranded circular DNA virus belonging to the Circoviridae family[1]. PCV2 is recognised as the causative infectious agent of postweaning multisystemic wasting syndrome[2] (PMWS) and is associated with a number of other porcine diseases[3].

Three phylogenetic groups of PCV2, namely PCV-2a, PCV-2b and PCV-2c, have been identified[4]. The vast majority of PCV2 viruses isolated belong to PCV-2a or PCV-2b genogroups whereas to date, only three PCV-2c isolates have been retrospectively identified from archive samples[4]. Phylogenetic characterisation requires DNA sequencing and is based on the proportion of nucleotide site differences between compared isolates. The PCV2 ORF2 (encoding viral capsid protein) gene has been suggested as a suitable candidate for performing phylogenetic analyses[4]. The aim of this study was to develop a minor groove binder (MGB) PCR assay to discriminate between the predominant PCV-2a and PCV-2b phylogenetic groups and assess its application as a molecular tool for the characterisation of PCV2 isolates.

Materials and Methods

MGB assays were designed against signature sequence motifs that discriminate between PCV-2a and PCV-2b genogroups using Qiagen web-based software (Qiagen Ltd., Crawley, UK). ORF2 DNA of known PCV-2a and PCV-2b isolates was cloned into TOPO pCRII cloning vectors (Invitrogen, Paisley, UK) and confirmed by DNA sequencing. The cloned material was used as positive control template DNA in subsequent studies. Forty cycles of real-time PCR were perfomed on a Mini-Opticon instrument (Bio-Rad, Hemel Hempstead, UK) in 25µl reaction volumes using 2µl of template DNA and JumpStart Taq Readymix (Sigma-Aldrich, Gillingham, UK). The assay was run with 3mM Mg^{2+}, 0.5µM primers at a Ta of 50°C. The specificity of the probes was assessed by screening against six known PCV-2a and PCV-2b isolates (Table 1). DNA extraction was performed using the MagNA Pure LC Total Nucleic Acid Isolation kit (Roche, Burgess Hill, UK). All samples were run in duplicate.

Results

A FAM-labelled probe was designed against an AAAATC signature sequence motif exclusive to the ORF2 of PCV-2a genogroup isolates. The probe was successfully incorporated into an MGB assay (Fig. 1). When the assay was applied to virus samples, all six isolates belonging to the PCV-2a genogroup were identified as positive while the PCV-2b isolates were all negative.

The results of the PCV-2b genogroup specific MGB assay are shown in Fig. 2. A TET-labelled probe was designed to recognise a CCCCGC nucleotide sequence within the ORF2 of isolates belonging to the PCV-2b genogroup. The assay successfully identified all six PCV-2b genogroup viruses, while PCV-2a isolates were all negative.

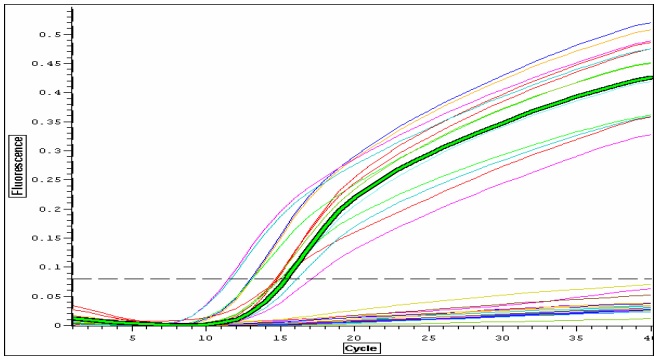

Fig. 1 Fluorescence (FAM) vs cycle number for PCV-2a MGB assay run against cloned DNA from a PCV-2a isolate and DNA extracted from six known PCV-2a and PCV-2b viruses.

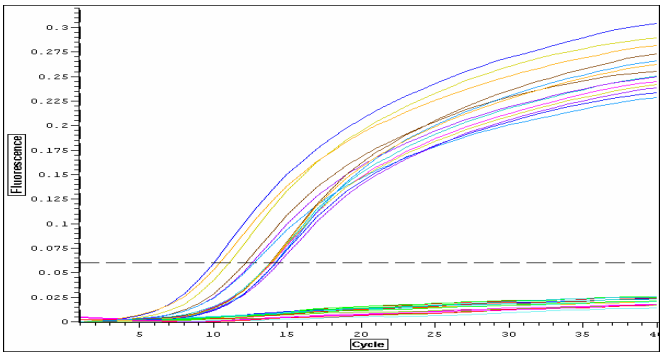

Fig. 2 Fluorescence (TET) vs cycle number for PCV-2b MGB assay run against cloned DNA from a PCV-2b isolate and DNA extracted from six known PCV-2a and PCV-2b viruses.

Table 1 Details of PCV-2a and PCV-2b isolates screened by the MGB assay.

Isolate	*Country of Origin (year isolated)*	*Genogroup*
1247	*Sweden (1993)*	
1010	*Canada (1997)*	
5549	*North. Ireland (1997)*	*PCV-2a*
1155	*Denmark (1999)*	
1117	*U.S.A. (1999)*	
10557	*North. Ireland (2003)*	
48285	*France (1997)*	
1452/3	*Sweden (2004)*	
9367	*Italy (2000)*	*PCV-2b*
1206	*Belgium (2000)*	
H2755	*Hungary (2000)*	
1127	*North. Ireland (2004)*	

Discussion and Conclusions

Minor groove binder (MGB) probes are short oligonucleotide probes that can function as a reporter in real-time PCR. The use of probe-based assays offers a number of advantages over contemporary PCR. This includes the specificity that MGB probes confers when compared to SYBR Green assays. The use of MGB probes also facilitates melt curve analysis post amplification which provides additional information regarding probe-target homology.

While the FAM probe was designed against a nucleotide sequence exclusive to the PCV-2a genogroup, the CpG motif recognised by the PCV-2b TET probe is similar to that found in PCV-2c genogroup. Therefore the specificity of the PCV-2b assay must be assessed against a target belonging to the PCV-2c genogroup in the future. From the present results it can be stated that MGB PCR assays that can distinguish isolates of the PCV-2a genogroup from those belonging to the PCV-2b/-2c genogroups have been developed.

Future work will include screening a range of PCV2 isolates and clinical samples in both assays. The potential of multiplex assay development will also be explored by replacing the TET labelled probe with a yakima yellow fluorophore.

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GENETIC DIVERSITY OF BOVINE VIRAL DIARRHOEA VIRUS ISOLATES FROM ITALY

Monica GIAMMARIOLI[1], Elena CANELLI[2], Sara CIULLI[3], Elisabetta ROSSI[1], Gian Mario DE MIA[1]

[1]Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Perugia, Italy;
[2]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy;
[3]Università di Bologna, Facoltà di Medicina Veterinaria, Ozzano Emilia (BO), Italy

Keywords: BVDV, genetic typing, molecular epidemiology, pestivirus

Introduction and objectives

Bovine viral diarrhea virus (BVDV) belongs to the genus *Pestivirus* with classical swine fever virus and border disease virus in the Family *Flaviviridae*. Presently two species of BVDV are recognised. BVDV-1 infection occurs worldwide involving mainly respiratory, reproductive and enteric organs. BVDV-2 causes similar clinical signs as BVDV-1, except that infection with highly virulent isolates may lead to fatal haemorrhagic syndrome. Genetic typing of BVDV has usually been performed using sequences from the 5'-UTR, N^{pro} and E2 regions. Sequence analysis of 5'-UTR can distinguish BVDV-1 and BVDV-2 and can subdivide BVDV-1 into at least 15 genetic groups [4, 5]. Genetic typing of BVDV-2 isolates has not been as extensive and at present, only 2 to 4 genetic groups of BVDV-2 have been described.

Studies on the prevalence of BVDV in Italy have been conducted providing evidence of circulation of 9 BVDV-1 subtypes [1, 2, 3].BVDV-2 circulation has been reported in cattle as well. To better define the genetic pattern within BVDV Italian isolates we studied a broad range of BVD viruses. Our genetic study has been based on the 5'-UTR supported by selected comparison within the N^{pro} coding region.

Materials and methods

A total of 111 virus isolates were sequenced in this work, additional sequences of other Italian BVDV isolates were acquired from the GenBank database. The viruses analyzed in this study were collected during the period 1995-2009 from 12 Italian regions. They were from cattle (n=106), sheep (n=4) and buffalo (n=4), mostly originated from farms located in northern Italy which is characterised by the highest cattle population density in the country. The genetic heterogeneity of the Italian BVDV viruses was investigated by phylogenetic analysis of partial 5'-UTR and for selected viruses, of the genomic region encoding autoprotease N^{pro}. Nucleotide sequences were determined by cycle sequencing three independent cDNA clones. Sequences were aligned using the Clustal X (version 1.83) analysis program and were proof read using the BioEdit version 7.0.0. Phylogenetic trees was calculated using the MEGA (version 3.1) program package based on the neighbor joining algorithm. The robustness of the phylogenetic analysis and significance of branch order were determined by bootstrapping method.

Results

Five isolates were typed as BVDV-2. The remaining isolates were typed as BVDV-1 and belonged to 10 distinct subtypes namely respectively BVDV-1a (n=12), BVDV-1b (n=47), BVDV-1d (n=4), BVDV-1e (n=26), BVDV-1f (n=5), BVDV-1g (n=4), BVDV-1h (n=7), BVDV-1j (n=1), BVDV-1k (n=2) and BVDV-1l (n=1). To confirm the grouping found in the 5'-UTR, we analysed in the N^{pro} region 19 viruses selected on the basis of their bootstrap value. The resulting phylogenetic tree showed that these viruses were clustered in the same phylogenetic branches as for the tree based on the 5'-UTR, with similar bootstrap values. Non-bovine isolates were all typed as BVDV-1. No relationship was observed between the geographic origins of the viruses and their phylogenetic clustering, with the exception of BVDV-1b, which is clearly predominant in northeastern Italy

Discussion and conclusions

The aim of this work was to study the genetic variability of a broad range of BVD viruses circulating in Italy. For this purpose an extensive collection of 111 BVDV isolates collected all over the country, has been investigated by genetic typing, providing further evidence for the heterogeneity of BVDV.

At the subgroup level, pair wise similarity and cluster analysis provided a clear-cut assignation to 10 distinct subtypes of 106 isolates typed as BVDV-1. Most cattle farms were infected by the predominant BVDV-1b and BVDV-1e isolates, the others genotypes occurred only sporadically. The results also provided evidence for circulation of the BVDV-1l subtype, which has been never shown before in Italy. Five field viruses were typed as BVDV-2, nevertheless the emergence of BVDV-2 in the field is very uncommon supporting the hypothesis of a iatrogenic infection in relation with contaminated vaccines. In summary, the results presented in this work revealed a high BVDV genetic heterogeneity in Italy. This is the result of the absence of any BVDV systematic control measures. Indeed, in Italy there is no BVDV national control program and the management practices such as cattle trade and movement, expose cattle herds to a high risk of introduction of BVDV infection as well as of new genetic variant as a consequence of a high diversity of BVDV.

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GENETIC AND ANTIGENIC CHARACTERIZATION OF A NOVEL PESTIVIRUS GENOTYPE

Monica GIAMMARIOLI[1], Severina Anna LA ROCCA[2], Cristina CASCIARI[1], Elisabetta ROSSI[1], Falko STEINBACH[2], Gian Mario DE MIA[1]

[1]Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Perugia, Italy;
[2] Department of Virology, Veterinary Laboratories Agency, Addlestone, UK

Keywords: antigenic typing, BDV, genetic typing, pestivirus

Introduction and objectives

Border disease virus (BDV) belongs to the well recognized *Pestivirus* genus, within the family *Flaviviridae*. Genetic analysis of pestiviruses isolated from sheep of continental Europe have led to the proposal that BDV isolates can be phylogenetically allocated into 6 genotypes, namely BDV-1, BDV-2, BDV-3 and BDV-4 [1, 5] and recently BDV-5 and BDV-6 [3]. In Italy, BD-like syndromes were observed in the southern regions since the 1990s and several pestiviruses isolated from lambs and kids, previously characterized as BDV, were subsequently recognized as BVDV-2 by genetic typing [4]. The molecular analysis of an Italian caprine BDV strain provided evidence for the presence of an atypical pestivirus which could represent the first member of a putative novel pestivirus subgroup [2]. As an extension of this study, a comparative analysis of ovine pestivirus strains isolated since has been performed in order to characterize genetically and antigenically originating from neighbouring geographical regions.

Materials and methods

Five pestiviruses, four isolated from sheep and one from goat, were included in this study. Four viruses were from flocks with clinical cases of abortion which occurred in central Italy between 2002 and 2005, one virus was isolated from a persistently infected sheep. Clinical samples detected positive by immuno-fluorescence test for the presence of pestiviruses were tested by RT-PCR. Parts of 5' untranslated region (5'UTR) and the N^{pro} coding region were successfully amplified. Both fragments were used for a phylogenetic study by sequencing three independent cDNA replicates.

All the isolates were also typed antigenically in binding assays with a panel of pestivirus specific mAbs directed against NS2/3, Erns and E2 proteins.

Results

The analysis of 5'UTR and N^{pro} sequences revealed that the pestiviruses could be allocated within the BDV species but interestingly could not be clustered with sequences obtained from GenBank database. Indeed these isolates significantly differ from all the ovine pestiviruses previously described providing evidence for the presence of a novel genetic subgroup. In the N^{pro} region the percentage of identity of pairwise evolutionary distances within the subgroup range was 77.5-100% compared to 69.6-76.4% between subgroups. On this respect the highest identities were found with isolates belonging to the BDV-3 and BDV-6 subgroups. All the 4 isolates react with a distinct set of mABs against E2, Erns and NS2/3. In particular against the BDV-E2 WS381, WS384 epitopes and the BDV-Erns WS371 epitope. The isolates are also greatly reactive for Erns WA433 and WA434 and NS2/3 WS437, that are typical for BVDV2, and E2 WB166 that is typical for BVDV1.

Discussion and conclusions

The ovine pestiviruses analysed in this study formed a separated cluster and could be allocated in a distinct genetic subgroup. On this basis we suggest to classify this set of isolates as subgroup BDV-7. Thus, actually BDV isolates can be grouped by phylogenetic analysis into at least seven major subgroups. It is very likely that pestiviruses belonging to this novel group were endemic in this central area of Italy and we cannot exclude the possibility that strains related to this genotype are still circulating because only few clinical samples from different sheep flocks were analysed. The results of the phylogenetic analysis on the Italian viruses in combination with other isolates classified as BDV, points out the inconsistency of *Pestivirus* nomenclature and the need for armonisation. The mAB reactivity pattern for these isolates also indicated that these BDV strains form a different group. More systematic studies should be done to analyse the diversity of these ovine/ceprine pestiviruses.

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PHYLOGENETIC ANALYSIS OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS ISOLATES FROM SLOVAKIA

Anna JACKOVA, Michaela NOVACKOVA, Stefan VILCEK

University of Veterinary Medicine, Kosice, Slovakia

Keywords: EU genotype, phylogenetic analysis, porcine reproductive and respiratory syndrome virus, PRRSV

Introduction and objectives

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, positive-stranded RNA virus with a relatively high level of sequence variation, especially between European and the American-type strains. The European isolates were considered to be less variable than the American strains. However, recently significant differences were detected in the Eastern European strains supporting the definition four genetic subtypes of the European genotype (Stadejek et al., 2006). However, according to new sequence information from the Russian Federation, the division of European genotype PRRSV-1 into three subtypes was proposed: a panEuropean subtype 1 and East European subtypes 2 and 3 (Stadejek et al., 2008). Also the Eastern European strains, from Belarusia, exhibited extreme ORF7 size polymorphism, ranging from 375 nt to 393 nt (Stadejek et al., 2006). All previous studies covering the time period 1989-2005 characterized the nucleocapsid protein as one of the most conserved PRRSV proteins (Drew et al., 1997; Forsberg et al., 2002).

The aim of this work was to detect and genetic characterize PRRSV strains from Slovakia by comparing their partial ORF5 sequences and ORF7 sequences and to analyze their relationship to the other European and American isolates.

Materials and methods

Forty clinical samples (serum, lymphatic node, lung) were obtained from eight pig farms in Western Slovakia from wasted animals, some of them showing respiratory problems. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer´s manual. The cDNA was prepared using gene specific primer and SuperScript reverse transcriptase (Invitrogen). Nested RT-PCR was used to amplify ORF5 and ORF7 regions with outer and inner primers according to Drew et al. (1997) and Oleksiewicz et al. (1998).

Purified PCR products were sequenced using automatic sequenser ABI PRISM. Both strands of the ORF5 and ORF7 PCR products were sequenced with the same primers as used for the nested PCR amplification. Partial ORF5 sequences (432 nt) and entire ORF7 sequences were assembled using SeqMan and MegAlign program from DNASTAR package.

Results

We detected PRRS virus by nested RT-PCR in ten samples from three different farms. The occurrence of PRRSV was indicated by respiratory problems of pigs, supporting by pathologic findings, and pathologic changes in lungs.

The nucleotide sequences of ten Slovak isolates were aligned and compared to each other and to selected European and American strains deposited in GenBank.

By phylogenetic analysis of partial ORF5 and ORF7 we found out that Slovak isolates clustered into three genetic groups. In the nucleotide alignment were observed identical mutation in isolates of first phylogenetic group, alike of second and third phylogenetic groups. All Slovak isolates were typed as EU genotype, subtype 1.

The comparison of nucleotide sequences identity Slovak isolates to each other showed 83.6 - 100 % similarity in ORF5 and 87.3 - 100 % in ORF7. We observed high nucleotide sequences identity of Slovak isolates to European reference strain Lelystad, 90.7 – 93.3 % in ORF7 and 85.2% - 89.6% in ORF5.

Similar, high nucleotide identity Slovak isolates was observed with Czech strain KT (GenBank EU071226) 93.8% in ORF7, lower 89.4% in ORF5.

By comparison Slovak and Poland strains, the highest nucleotide identity was observed between Poland strain Che-46 (GenBank DQ324673), as subtype 1, 91.7 % in ORF7 and 88% in ORF5.

On the other hand, Lithuania strains of subtype 2 and Belarusian strains of subtype 3 were identical with Slovak isolates only in range 82.4 – 86.9 % in ORF7 and 77.3 – 83.1 % in ORF5.

Slovak isolates compared with American reference strain VR-2332 revealed nucleotide sequences identity 64.8 - 66.2 % in ORF5 and 61.6 – 65.9 % in ORF7 region.

Results obtained by the genetic typing of Slovak isolates in partial ORF5 confirmed genetic analysis in ORF7 region. Moreover, it was observed extreme ORF7 size polymorphismus – 399 nt (132 amino acids and stop codon) in two isolates. The sequences of other Slovak isolates had an ORF7 size 387 nt, identically with European reference strain Lelystad and other the East European strains, for example Poland strains (GenBank DQ324673; DQ324688; DQ324715) and Russian strain (GenBank EU071257).

Discussion and conclusions

The results of this study revealed that Slovak isolates were clustered into three different phylogenetic groups, showing herd specific isolates, similarly in partial ORF5 and entire ORF7 region. All of them belong to EU genotype, subtype 1, according to the nomenclature by Stadejek et al. (2006).

Until now, the largest nucleocapsid protein described by Stadejek, et al. (2006), had 130 amino acids (393 nt) in the Belarusian PRRSV sequences, belonging to subtype 2. Two Slovak isolates exhibited exceptionally size polymorphism 399 nt (132 aa) – the longest described so far.

Acknowledgements

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CHARACTERISATION OF A NON-PROTECTIVE AND A NON-PATHOGENIC INFECTIOUS RABBIT HAEMORRHAGIC DISEASE-LIKE VIRUS

Ghislaine LE GALL-RECULE[1], Françoise ZWINGELSTEIN[1], Marie FAGES[2], Stéphane BERTAGNOLI[2], Jacqueline GELFI[2], Stéphane MARCHANDEAU[3]

[1]AFSSA (French Agency for Food Safety), Avian and Rabbit Virology, Immunology, and Parasitology Unit, Ploufragan, France;
[2]ENVT (National Veterinary School of Toulouse), UMR INRA/ENVT 1225, Toulouse, France;
[3]ONCFS (National Game and Wildlife Agency), Research Department, Nantes, France.

Keywords: lagovirus, non-pathogenic RHDV-like strain, phylogeny, rabbit

Introduction and objectives

Rabbit haemorrhagic disease (RHD) is a highly infectious and fatal disease of the European rabbits (*Oryctolagus cuniculus*), first described in China in 1984. The etiological agent of the disease, the *Rabbit haemorrhagic disease virus* (RHDV), is a non-enveloped single-stranded positive-sense RNA virus belonging to the family *Caliciviridae,* genus *Lagovirus.* Confirming previous serological results, the existence of non-pathogenic RHDV strains has been established in 1996, when a non-lethal RHDV-like strain genetically distinct from RHDV and called Rabbit calicivirus (RCV) has been isolated in a rabbitry in Italy (*1*). In the same way, lagovirus RNA sequences were characterised in RHDV-positive sera of healthy English wild rabbits and of commercial rabbits collected before the first occurrence of RHD (*4*). This result confirmed previous serological evidence of the presence of such viral strains in healthy rabbits. Partial RNA sequences are closely similar to sequences of virulent RHDV strains except one genetically distinct sequence (Ashington strain) characterised from a dead wild rabbit showing typical clinical signs of RHD a priori. Recently, a new divergent strain of RHDV (Lambay strain) close to the Ashington strain but collected from two healthy wild rabbits was described (*2*), and a second one named "RCV-A1" was identified in the intestinal tissues of healthy young wild rabbits in Australia (*5*).

Competition between non-pathogenic and pathogenic strains and the possible role of the non-pathogenic strains in reducing the impact of RHD has been discussed by several authors on the basis of partial crossed protection between strains. However, a recent study showed that antibodies produced in response to some RHDV-like strains may not protect against RHDV (*3*). Thus, study of the competition between circulating strains needs to take their diversity into account.

The objectives of the present work were to isolate from healthy rabbits an infectious lagovirus related to RHDV and to establish phylogenetic relationships with other lagoviruses. We have also investigated the protection induced by the antibodies towards a lethal RHDV infection and the absence of pathogenicity for immunologically naïve rabbits.

Materials and methods

Sera were collected twice one week apart (D0 and D7) from sixty 10-week-old fattening New Zealand White rabbits showing no apparent sign of disease. VP60-RHDV ELISA was used to screen for the presence of RHDV (*3*). Positive sera were sent to IZSLER (Italy, Dr Capucci) to be screened with anti-RHDV isotype ELISA tests that are specific to pathogenic RHDV strains antibodies.

Two protection studies were performed on rabbits exhibiting a protective level of antibodies: i) 3 rabbits were challenged with a RHDV standardized inoculation and 2 rabbits were placed into an adjacent cage in order to favour an infection by a natural route, ii) 10 rabbits were kept in hutches and were fed with grass taken from a RHD contaminated area, in order to simulate a natural RHDV outbreak.

For virological analyses, a piece of small intestine was collected from 30 seronegative or slightly seropositive rabbits and disrupted. RNAs were extracted and reverse transcribed. For the initial screening, several RHDV PCRs were used and positive cDNAs were subjected to a full-length VP60 capsid protein PCR by using a high-fidelity DNA polymerase. The DNA sequence was determined for several PCR products. Phylogenetic relationships were inferred using VP60 gene sequences available in databases from representative pathogenic RHDV and antigenic variant "RHDVa" strains, the non-pathogenic RCV and RCV-A1 strains, and the highly divergent Ashington strain (partial sequence). Phylogenetic analyses were conducted using MEGA version 3.1 software for phenetic and cladistic methods.

An experimental viral transmission trial was performed with two 10-week-old SPF New Zealand White rabbits to verify the absence of the RHDV-like pathogenicity, i) the first SPF rabbit was placed into a cage with three seropositive 9-week-old healthy rabbits taken from the contaminated rabbitry, ii) the second SPF rabbit was placed into a separated cage and was inoculated by the oronasal route with filtered supernatant of a pool of small intestine in which RHDV-like RNAs were characterised. Blood samples were collected twice 5 days apart (D5 and D10), then at the sacrifice (D14). Detection of antibodies was performed using the VP60-RHDV ELISA and virological analysis were realised by RT-PCR.

Results

The VP60-RHDV ELISA test revealed that 33% of the sera of fattening rabbits were seropositive at D0 and 83% one week later, indicating that an active infection was in progress without mortality. Conversely, the results obtained with the isotype ELISA tests revealed that these antibodies were not specific to those induced by pathogenic RHDV.

During the protection experimental trial, the 3 challenged rabbits died within 2-4 days, whereas the 2 rabbits placed in an adjacent cage died within 4-6 days. These levels and delays of mortality are compatibles with a RHDV experimental infection on immunologically naïve rabbits. Regarding the 10 rabbits kept in hutches, a RHDV outbreak occurred 5 days later and 9 rabbits died within 4 days. The sequencing result confirmed the presence of a RHDV close to RHDV commonly characterized in France during the same period.

Virological analyses characterised lagovirus RNAs in the fattening rabbits. We have determined the complete "06-11" VP60 gene sequence: it is 1734 nt long (578 aa long) and when compared to RHDV has a 6 nt deletion in length (aa 309-310). A similar deletion is present at the same location in the RCV and Ashington strain genomes. The average nucleotide identities between the "06-11" strain and the VP60 genes of RHDV is 83 %, of RCV is 85 %, and of Ashington is 93.8 %.

Phylogenetic analyses showed that the rabbit lagoviruses were divided into 3 major groups (RDV-A1 as outgroup), i) a group comprised of the pathogenic RHDV strains further separated into viruses related to the RHDV and the "RHDVa", ii) the non-pathogenic Italian RCV strain, iii) a group comprised of the Ashington strain and the non-pathogenic "06-11".

The experimental trial performed on SPF rabbits showed the presence of antibodies from D5 in the contact SPF rabbit and from D10 in the inoculated SPF rabbit. No sign of RHD was detected during the assay and at necropsy. RT-PCR analyses were positive for 2/3 fattening rabbits and for the 2 SPF. In addition for the contact SPF rabbit, the identified RNA sequence was identical to that of "06-11' strain.

Discussion and conclusions

We have characterised and have determined the entire sequence of the gene encoding the capsid protein of a new divergent strain of RHDV different to RCV and RCV-A1 but close to Ashington and Lambay strains. The "06-11" strain was isolated from healthy domestic rabbits suggesting that such viral strains circulate in both wild and domestic rabbits.

At the opposite of the RCV and some RHDV-like strains, we have demonstrated that the detected RHDV antibodies do not protect rabbits from a RHDV infection and confirmed the first observations established from wild rabbits (*3*). By showing for the first time that a RHDV-like strain can be experimentally transmitted through contact from naturally infected healthy rabbits to immunologically naïve rabbits without causing disease, we have demonstrated that this strain is infectious but non-virulent. In other respects, we have succeeded to isolate and to amplify it in rabbits.

Search of non-pathogenic RHDV-like strains in French wild rabbits is in progress. In addition, we are sequencing the full length genome of the "06-11" strain to obtain more genetic information, notably regarding potential recombination events, in order to contribute to the knowledge of the lagovirus diversity.

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DETECTION OF PORCINE CIRCOVIRUS IN RODENTS

Márta LŐRINCZ[1], Attila CSÁGOLA[1], Imre BIKSI[1], Levente SZEREDI[2], Ádám DÁN[2], Tamás TUBOLY[1]

[1] *Szent István University, Faculty of Veterinary Science, Budapest, Hungary,* [2] *Central Veterinary Institute, Budapest, Hungary*

Key words: PCV2, rodents

Introduction and objectives

Circoviruses have small (~2-kb), covalently closed, circular, single-stranded DNA (ssDNA) genomes within non-enveloped icosahedral virions of 17–26.5 nm. Members of the family Circoviridae are divided into two genera, namely the *Gyrovirus* and the *Circovirus* genus. Several different viruses belonging into the family have been described so far, including two porcine circoviruses PCV1 and PCV2, interestingly all other known circoviruses were detected in birds.

Data about the presence of PCV in mammalian species other than swine are controversial. The role of mice as possible reservoirs for the virus and source of swine infections cannot be excluded as Kiupel et al. (2001) demonstrated that PCV2 could replicate in mice after simultaneous infections by the intranasal and intraperitoneal routes. On the other hand, another researcher group could not detect the PCV2 genome by *in situ* hybridization in tissues of experimentally infected mice. Studies by Cságola et al. (2008) indicated that mice were able to transmit PCV2 to uninfected mice after oral or parenteral infections, under laboratory conditions.

The objective of this study was to investigate the presence of PCV in rodents (mainly mice and rats) under field conditions.

Materials and methods

Twenty mice (18 *Mus musculus* and 2 *Mus agrarius*) and 21 rats (*Rattus norvegicus*) were collected at PCV2 infected farms and grouped according to the location and time of collection. Twenty-five samples of mice (yellow-necked mouse: *Apodemus flavicollis* and mouse: *Mus musculus*) and common voles *(Microtus arvalis)*, from areas not directly connected with PCV2 infected farms were also sampled and tested.

DNA from tissues (lungs, liver, kidneys, lymph nodes, spleen, fetus and muscles) was extracted by standard methods and circovirus DNA was amplified by polymerase chain reaction (PCR) using different primers:

- PCV-ORF1 specific primer pair (Fenaux et al., 2000),
- nested, degenerate primer pairs (Halami et al., 2008),
- PCV2-Cap gene specific (Cságola et al., 2006),
- nested system based on PCV1 and PCV2 specific primers (Cságola et al., 2006; Fenaux et al., 2000).

Amplicons from each group were selected, sequenced and compared with each other and available GenBank data.

Results

PCR reactions indicated that rodent samples collected from outside PCV2 infected pig farms were negative for circoviruses. Among the rat and mice samples originating from pig farms several carried PCV2 sequences, namely 13 mice and 4 rats. The DNA sequence analysis showed that the rodents carried the same PCV2 as the pigs on the given farm, although low levels of nucleotide differences could be detected. Interestingly none of the fetuses of the 3 pregnant mice and 9 pregnant rats were PCV positive.

Discussion and conclusions

33.3% of mice and 23,8% of the rats from pig farms were PCV2 positive (the swine samples were 80% positive). According to the results PCV2 can be present in organs of rodents (mice and rats) on PCV2 infected pig farms, and serve as carriers of the virus, but their role in transmitting PCV2 between premises could not be proven, as mice outside farms were negative for PCV2. Attempts to identify rodent specific circoviruses were not successful.

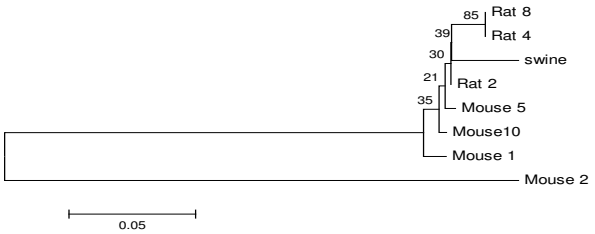

Figure 1. Comparison of PCV2 sequences generated in the study.

Table 1. PCV2-specific PCR positive animals and samples.

Group	# of animals	# of PCV2 positive / total				
		lung	liver	kidney	L+S*	pooled tissues
1 (mice)	4	1/4	0/4	1/4	1/4	4/4
2 (mice)	5	1/5	0/5	0/5	0/5	1/5
3 (mice)	1	0/1	0/1	0/1	0/1	1/1
4 (mice)	4	0/4	0/4	0/4	0/4	4/4
5 (mice)	6	0/6	0/6	0/6	0/6	6/3
6 (rat)	9	1/9	1/9	1/9	1/9	9/3
7 (rat)	12	1/12	1/12	2/12	1/12	12/2
8 (swine)	10	8/10	0/10	0/10	8/10	0/10

L+S*: lymph node + spleen

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MOLECULAR ANALYSIS OF EQUINE HERPESVIRUS-1 STRAINS ISOLATED IN THE LAST 30 YEARS IN HUNGARY

Péter MALIK, Ádám BÁLINT, Ádám DÁN, Vilmos PÁLFI

Central Agricultural Office Veterinary Diagnostic Directorate, Budapest, Hungary

Keywords: equine herpesvirus 1, herpesvirus, PCR

Introduction and objectives

Equine herpesvirus-1 (EHV-1) is a major pathogen of horse with world-wide distribution causing respiratory disease, abortion in mares and occasionally neurological syndromes (3, 4). The virus infects not only horses, it also occurs in different species among *Equidae* (1). A comparison of full genomes of different EHV-1 strains showed that several ORFs contain particularly polymorphic regions. In a study single nucleotide polymorphisms (SNPs) in ORF68 were found to be associated with geographical origin, rendering it a good grouping marker (2). This ORF is homologous to herpes simplex virus type 1 (HSV-1) Us2 and related genes of other alphaherpesviruses (2). In the present study we aimed at establishing groups from our 35 isolated EHV-1 strains based on the nucleotide sequence of ORF68.

Materials and methods

The 35 EHV-1 strains used in this study were isolated between 1977 and 2008. The isolates were propagated in rabbit kidney cells (RK-13). DNA was purified from tissue cultures with visible cythopathogenic effect using High Pure Viral Nucleic Acid Purificatiopn Kit (Roche).

Four primers were designed targeting the polymorphic part of ORF68, amplifying a 764 bp long product. The amplicons were generated with RedTaq Polymerase (Sigma). The PCR products were sequenced with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an automatic ABI PRISM 3130 genetic analyser (Applied Biosystems). Sequences were assembled and edited using the BioEdit v.7.0.7 and the DNASTAR 7 (Lasergene, WI, USA) software package.

Results

In the study mentioned above (2), six groups were defined among the isolates originated from Europe, Australia and America. 23 of the 35 strains could be classified into the previously described six groups, but no group 1 and 6 strain was found among our isolates. Moreover, on the basis of different SNPs we could establish two additional groups containing 5 strains each. We also found two isolates that could not be classified into any of the eight groups. The new groups may contain rare strains, because they have not been found in previous studies. However, they were present in several Hungarian studs and could be isolated from different outbreaks over the years.

Our study confirmed that the nucleotide sequence of ORF68 cannot be associated with the pathogenicity of virus strains because the highly virulent ARMY 183 and RacH, a vaccine strain were in the same group.

Discussion and conclusions

The results of our study suggest that the genetic variability among EHV-1 is greater than previously suspected, and new groups can be identified when the increasing number of isolates are analysed. Although virus strains were isolated in Hungary, based on our data, they can be originated from the neighbouring countries so the geographical specificity is applicable for bigger regions not only for countries. According to the original study two groups were distinguished by the number of G residues in a homopolymeric tract (nt 732 to 739). However, the difficulty of accurately sequencing GC-rich regions makes grouping uncertain.

Our results show that ORF68 is a useful grouping marker, and though it cannot be used to distinguish strains based on their pathogenicity, this method can be used in epidemiological studies. Based on the sequencing of strains isolated from the same stud at different time points, it may be determined whether recurrence or reinfection occured.

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INCIDENCE OF PORCINE CIRCOVIRUS TYPE 2 (PCV2) AND *TORQUE TENO* VIRUS (TTV) GENOGROUPS IN PIGS FROM POST-WEANING MULTISYSTEMIC WASTING SYNDROME (PMWS)-AFFECTED AND NON-PMWS-AFFECTED FARMS IN ARCHIVAL UK SAMPLES

Michael J. McMenamy[1], Irene McNair[2], Catherine Duffy[1], John McKillen[2], Nuala Fee[2], Catherine Mulholland[2], Brian Adair[2], Gordon Allan[2]

[1]*Department of Veterinary Science, The Queen's University of Belfast, Belfast, Northern Ireland BT43SD;*
[2]*Veterinary Sciences Division, Agri-Food and Biosciences Institute, Belfast, Northern Ireland BT4 3SD*

Keywords: PCV2, PMWS, TTV

Introduction and objectives

Postweaning multisytemic wasting syndrome (PMWS) is now recognised as an economically important epizootic where porcine circovirus type 2 (PCV2) is acknowledged as the essential infectious agent of PMWS[1]. At present 3 PCV2 genotypes have been characterised[4]. Currently PCV-2b is mainly evident in pigs suggesting a chrological shift in genotypes. Here we briefly investigate those PCV2 genotypes evident in archival serum and tissue samples recovered from pigs from PMWS-affected and non-PMWS-affected farms in the UK, collected at regular intervals between 2000 to 2004.

In many cases where PCV2 is detectable it has not resulted in the development of PMWS[2]. It has been suggested that *Torque teno* virus genogroup 1 (g1-TTV), which is from the same viral family as PCV2, may be a potentiating co-factor of PMWS although not a directly causative agent[2]. Furthermore, a recent Spanish study noted that pigs suffering from PMWS exhibited a 1.25 times higher incidence of infection with *Torque teno* virus than those pigs unaffected by PMWS included in their survey[3]. It is possible that TTV might contribute to the immunologic dysregulation method of PMWS induction. Even though other potentiating co-factors have been suggested it would be prudent to further investigate the possible impact of TTV in tandem with PCV2 on the development of PMWS in swine. Here we investigate the incidence of PCV2 and TTV in the previously mentioned archival serum and tissue samples.

Materials and methods

171 sera and tissue samples including, lung, liver, kidney, sleen and lymph nodes were collected from pigs from UK farms know to be either PMWS-affected or non-PMWS-affected over a four year period (2000-04). Viral nucleic acids were extracted from 200µl of all samples using a MagNa Pure LC automated liquid handling system and the associated MagNA Pure LC Total nucleic acid extraction kit (Roche, Burgess Hill, UK). Samples were assessed for the presence of PCV2 by amplifying PCV2 specific DNA followed by gel electrophoesis[1]. Resultant PCV2 amplicons were sequenced, where possible, with BigDye Terminator v3.1 (Applied Biosystems, Cheshire, UK)[1]. The same samples were further assessed for the presence of g1-TTV using a set of outer primers that compose a nested set in real-time using QuantiTect SYBR Green PCR Master Mix (Qiagen, West Sussex, UK)[3]. Amplification was acheived using 40 cycles of 95℃ for 30 seconds, 52℃ for 30 ℃ seconds and 72 for 30 seconds followed by a melt-curve between 50-95℃, carried out on a Roche LC480 instrument (Burgess Hill, UK) in 25µl reaction volumes containing 2µl of template nucleic acid. g2-TTV was detected in the same manner again using a set of outer primers from a nested set kindly provided by Dr. Annette Mankertz (Robert Koch-Institut, Berlin, Germany) (data not published). In this case the annealing temperature of this primer set was increased to 60℃ whereas all other real-time PCR conditions remained the same.

Results

It was not always possibly to amplify sufficient ORF2 target material to permit sequencing however, efforts were made to sub-divide those PCV2 genomes detected into their appropriate genotypes based on current nomenclature[4]

The percentage incidence of those viruses detected was calculated (Table 1). This data gives some measure of the presence of each specified virus in those samples collected from farms either known to be affected or free from PMWS. It is important to note that this measure of incidence per virus or combinations of virus does not suggest that these samples are free from or harbour those viruses also included in this study.

Table 1. Percentage incidence of PCV2 and individual TTV genotypes alone and in combination from samples of PMWS-affected and non-PMWS-affected farms.

	PCV2 +VE	g1-TTV +VE	g2-TTV +VE	g1-TTV & g2-TTV +VE	PCV2 & TTV +VE
PMWS +VE	77%	61%	62%	42%	34%
PMWS -VE	69%	47%	54%	38%	28%

Discussion and conclusions

Sequencing of a selection of the PCV2 positive samples gave some idea of the chronological change in PCV2 genotypes encountered in this study. It has been suggested that the chronological shift in PCV2 genotypes may also have been accompanied by a change in pathogenicity, hence the interest in disease state and PCV2 genotype. Of those samples sequenced between the years 2000 and 2002, genotypes PCV-2a and PCV-2b were evident[4]. However from 2003 onwards and based on those PCV2 positive samples suitable for sequencing the genotype observed was consistently PCV-2b in character[4]. This is broadly consistent with what we have previously observed on the change in PCV2 genotypes over time.

Regarding the incidence of those viruses detected in all cases, the percentage was higher in samples recovered from pigs from PMWS-affected farms than in those that had not been affected by PMWS. Both genogroups of TTV were also evident in higher concentractions at the same time in pigs from PMWS-affected farms and although only slightly higher, it appears that pigs from PMWS-affected farms also harbour both PCV2 and TTV to a greater degree than those not affected. Interestingly there is only a marginal difference in the the percentage incidence of both TTV genogroups and the combined presence of PCV2 and TTV in both pigs from PMWS-affected and non-PMWS-affected farms.

Although further statistical analysis is required to assess the significance of this data (which is currently ongoing) the results possibly suggest that at least the presence of one or other of the TTV genogroups on their own could be linked to the onset of PMWS in PCV2 infected swine.

Acknowledgements

Samples used in this study were collected as part of two other previous surveys in conjunction with the University Of Warwick (UK) and the Meat and Livestock Commission (MLC, UK).

Thanks to Dr. Annette Mankertz for providing those primer sequences utilised in the detection and analysis of g2-TTV positive samples.

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MOLECULAR CHARACTERIZATION OF CANINE PARVOVIRUS TYPE 2 CIRCULATING IN GREECE

Vasileios NTAFIS[1], Efthychia XYLOURI[1], Iris KALLI[2], Katerina ADAMAMA-MORAITOU[2], Timoleon RALLIS[2], Theofanis KANELLOS[1], Costantina DESARIO[3], Nicola DECARO[3]

[1]Agricultural University of Athens, Faculty of Animal Science, Iera Odos 75, 118 55, Athens, Greece;
[2]Small Animal Hospital, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Staurou Voutyra 11, 54627, Thessaloniki, Greece;
[3]Department of Veterinary Public Health, Faculty of Veterinary Medicine of Bari, Strada per Casamassima Km 3, 70010 Valenzano (Bari), Italy

Keywords: canine parvovirus, CPV-2, dogs, variants

Introduction and objectives
Canine parvovirus type 2 (CPV-2) is the causative agent of acute hemorrhagic enteritis in dogs. Nowadays, the reports indicate that there are three antigenic variants circulating, CPV-2a, CPV-2b and CPV-2c, with the last being detected for the first time in 2000 in Italy. The aim of the present study was the molecular characterization of the CPV variants, circulating in Greece, during 2008.

Materials and methods
167 fecal samples from puppies with clinical symptoms compatible with acute hemorrhagic enteritis were collected from different regions of Greece. Detection of the pathogen was done by means of PCR (1), while minor-groove binder (MGB) probe assays were used for the characterization of the variants and the discrimination between vaccine and field strains (2, 3).

Results
84 out of 167 samples were positive, of which 81 were CPV-2a, 1 was CPV-2b and 2 were CPV-2c, the recently detected variant. Vaccine strains were not detected in any sample.

Discussion
The results indicate that CPV-2c variant is also present in Greece. Among the three variants detected, CPV-2a still prevails, unlike in other European countries (4).

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MOLECULAR EPIDEMIOLOGY OF RABIES VIRUS ISOLATES IN SLOVENIA

Danijela RIHTARIČ, Ivan TOPLAK, Urška KUHAR, Jože GROM, Peter HOSTNIK

[1]*University of Ljubljana, Veterinary Faculty, Institute for Microbiology and Parasitology, Virology Unit, Gerbičeva 60, SI-1115 Ljubljana, Slovenia*

Keywords: molecular epidemiology, phylogenetic analysis, rabies virus

Introduction and objectives

Rabies is caused by highly neurotropic viruses belonging to *Lyssavirus* genus within Rhabdoviridae family. The rabies virus genome consists of a non-segmented, single-stranded negative-sense RNA of about 12 kb. The genome encodes five viral proteins (3' N-P-M-G-L 5'). Phosphoprotein (P) in complex with the nucleoprotein (N) forms the nucleocapsid (NC). Matrix protein (M) plays a pivotal role in linking the NC structure with the envelope bilayer. The glycoprotein (G) is a type 1 trans-membrane protein that forms spikes on the viral particle surface and plays a crucial role in viral neurotropism and pathogenicity. RNA polymerase (L) has a role in transcription of the viral RNA (5).

Rabies virus is distributed worldwide and causes lethal viral encephalitis in a wide range of host species, including human. Rabies occurs in two different epidemiological forms: urban rabies, with the domestic dog as the main reservoir and transmitter, and sylvatic rabies, with different wildlife species acting as reservoirs (5). Oral vaccination against rabies is the most effective method for preventing rabies virus infection in wild animals. Program of oral vaccination of foxes in Slovenia has been running since 1988.

The *Lyssavirus* genus is divided into seven genotypes. Genotype 1 is known to be the most widespread and represents the classical rabies viruses including the majority field, laboratory and vaccine strains. Rabies related viruses, isolated in Africa belong to genotypes 2, 3, and 4. Viruses isolated in bats in Europe belong to genotypes 5 and 6, while Australian bat Lyssavirus isolates present genotype 7 (2). Rabies molecular epidemiology studies have recently gained relevance in Slovenia as a way to investigate the dynamics of transmission in the geographical and inter species area of knowledge.

A molecular epidemiological study was performed on 41 Slovenian isolates, which were collected between 1994 and 2009 from wild (fox, badger, marten) and domestic (cattle, dog, cat, horse) animals diagnosed as rabid. The study was based on the comparison of nucleotide sequences of the 3' terminal segment of the N gene, previously identified as one of its most variable regions.

Materials and methods

A total of 41 brain samples from 28 red foxes, 5 dogs, 3 badgers, 2 cattle, 1 cat, 1 marten and 1 horse were used for genetic analysis in this study. All samples were tested by fluorescent antibody test (FAT) and found to be positive for virus antigen, using commercial rabies anti-nucleocapsid conjugate (BioRad).

Total viral RNA was extracted from original host brain samples using QIAamp® Viral RNA Mini Kit (Qiagen, Germany) according to manufacturer's instructions. Extracted RNA was stored at -70 °C until analysis.

Reverse transcription (RT) with polymerase chain reaction (PCR) was performed in one tube (One-Step RT-PCR Kit, Qiagen, Germany) with primer set N1161P and N1579M to amplify 419 bp PCR product of the nucleo-phosphoprotein (N-P) gene segment (4). RT-PCR reaction was carried out in a volume of 50 µl. The reaction mixture included 5x PCR buffer, 10 mM dNTP mix, 400 nM of each primer, RT-PCR Enzyme mix and 4 µl of RNA. A thermocycling profile of 1 cycle of 30 sec at 50 °C, 1 cycle of 15 min at 95 °C, 40 cycles of 30 sec at 94 °C, 30 sec at 56° C, and 1 min at 72° C was employed followed by a final incubation at 72 °C for 10 min. The amplified products were visualized on 1,8 % agarose gel with ethidium bromide.

Purification and sequencing of amplified RT-PCR products was done by Macrogen Inc (Korea). Multiple alignments were created with Clustal X 1.83 program. Phylogenetic tree was constructed by the Neighbour-Joining method using Kimura 2-parameter for calculating pairwise distances in MEGA program version 4 (fig. 1). Statistical support for the tree was evaluated by 1000 bootstrap replicates. Sequence distances were computed using DNASTAR program (USA) and compared to the published sequences in GenBank database.

Results

A specific RT-PCR product (419 bp) was amplified and sequenced in all samples. Nucleotide sequences of the 246 nt long segment of the nucleoprotein gene were compared to the published sequences in GenBank database. Rabies isolates from Slovenia showed 96,3 – 100 % nucleotide homology to other European isolates and 91,9 – 93,5 % nucleotide homology to vaccine strains PV and SAD B19. Sequence comparison revealed 0 – 2,1 % nucleotide divergence between 41 Slovenian isolates. Phylogenetic analysis showed that all Slovenian isolates belong to genotype 1.

Discussion and conclusions

The result of this study confirmed that all rabies isolates from Slovenia belong to genotype 1. The phylogeny, determined in this report, based on 3' terminal nucleoprotein gene sequences of 41 Slovenian rabies virus isolates recovered from different animals in period 1994 – 2009, indicated the existence of single group of closely related rabies viruses, belonging to West Europe group. When compared with the same gene region of rabies isolates from all Europe, isolates from Slovenia showed 96,3 – 100 % nucleotide homology. Some Slovenian rabies virus isolates were closely related (100 %) to isolates from Italy and former Yugoslavia (1, 3). Slovenian isolates from this study are genetically divergent to vaccine strain SAD B19, which is in use for oral vaccination since 1988. The phylogenetic analysis and genetic diversity obtained in our study were consistent with other published data in which entire nucleoprotein or other genes were used (1, 2, 3).

This study revealed phylogenetic relationship between Slovenian and European field rabies virus isolates. For more detailed phylogenetic study a longer part of nucleoprotein gene will be sequenced and analyzed.

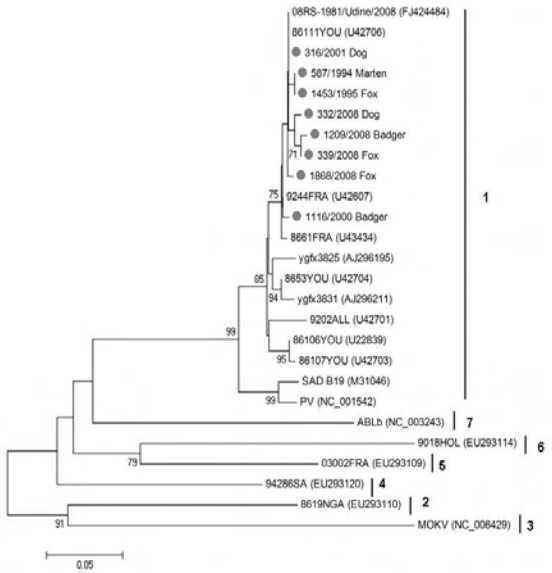

Figure 1: Phylogenetic relationships of representative isolates from Slovenia collected between 1994 and 2009 with other isolates from genotypes 1 – 7 are presented. Analysis was based on partial nucleoprotein gene sequences (246 nt), between positions 1175 and 1421, numbering in the genome position of vaccine strain SAD B19. Numbers at nodes indicate bootstrap values greater than 70 %.

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PHYLOGENETIC ANALYSIS OF SMALL-RUMINANT LENTIVIRUSES IN SHEEP FLOCKS / GOAT HERDS IN GREECE

Vassiliki SPYROU[1], Vassilios PSYCHAS[2], Georgios SOFIANIDIS[2], Vasia MAVROGIANNI[3], Emmanouil KALAITZAKIS[3], George FTHENAKIS[3], Charalambos BILLINIS[3,4]

[1]Department of Animal Production, Technological Educational Institute, Larissa, Greece;
[2]School of Veterinary Medicine, Aristotle University, Thessaloniki, Greece;
[3]Faculty of Veterinary Medicine, University of Thessaly, 224 Trikallon, Karditsa, Greece;
[4]Institute of Biomedical Research and Technology, 51 Papanastasiou, Larissa Greece.

Keywords: Greece, phylogenetic analysis, small ruminant lentiviruses

Introduction and objectives

Small ruminant lentiviruses (SRLVs), which include Maedi-visna virus (MVV) of sheep and caprine arthritis encephalitis virus (CAEV) of goats, are widespread throughout the world. According to a recently proposed nomenclature, based on 1.8 kb gag-pol and 1.2 kb pol sequences, the SRLVs are classified into four groups. Group A can be further subdivided into seven subtypes: A1-A7, where the subtype A1 is identified by the genetically and geographically heterogeneous MVVs. Group B refers to the CAEV type and comprises two distinct subtypes: B1 and B2. Groups C and D have been only recently identified on the basis of their great genetic divergence with the other two groups. Recently, direct evidence for natural sheep-to-goat and goat-to-sheep transmissions of particular subtypes (A4 and B1) of the virus has been shown (4). The aim of this study was the genetic characterization of small ruminant lentiviruses of sheep and goats in Greece and to determine the position of these strains in the current taxonomic classification.

Materials and methods

Blood and tissues samples (myocardium, lungs, mediastinal lymph nodes, ileum, mesenteric lymph nodes, liver, spleen, kidneys, mammary glands, supramammary lymph nodes, carpal and tarsal joints, brain with cervical spinal cord and chorioid plexus) were collected from ten sheep and six goats seropositive to SRLV in Greece (from: four sheep flocks, one goat herd and one mixed sheep and goat farm). Salient clinical signs in these 16 animals were pneumonia, progressive weight loss, mastitis and arthritis. Samples were examined for proviral DNA, viral RNA and histopathologically. Tissues were processed routinely for parafine embedding and 4-6 μm thick sections were cut and stained with haematoxyline and eosin. Proviral DNA and viral RNA were isolated using a DNA and a RNA isolation kit (Gentra Systems, Minneapolis USA), according to the manufacturer's instruction. Primers for a nested PCR specific for the gag-pol and the pol gene region were used to amplify a 1.8 kb and a 1.2 kb sequence, respectively (4). PCR products were gel-purified (QIAquick Gel Extraction Kit, Qiagen Ltd, West Sussex, UK) and sequence analysis was performed twice on the complete viral genome (MWG Biotech, Ebersberg, Germany) by using the forward and reverse PCR primers. All samples were analyzed twice and only high-quality sequences were used. Nucleotide sequences from others SRLVs isolates were retrieved from the GenBank. A phylogenetic and molecular evolutionary analysis was performed using the program MEGA 3.1 (1).

Results

The majority of the animals were in a poor body condition. Gross pathological and histological lesions characteristic of lentiviral infection were consistently present in the lungs and the mammary glands of the animals. Occasionally, lesions were also evident in the joints. Specifically, the lungs were swollen and failing to collapse when the thorax was opened; they were diffusely affected, most severely in the caudal lobes, with a "rubbery" consistency with gray-pink areas; on the cut surface whitish gray foci, about 2 mm in diametre, were observed, whilst some bronchi contained small amount of whitish fluid. The mediastinal lymph nodes were enlarged. The mammary glands were found to be of decreased size and firm consistency; mammary secretion was watery; supramammary lymph nodes were enlarged. Swelling of the carpal joint with thickened capsule was seen in some animals. Histologically, the alveolar septa were markedly thickened with mononuclear cells, mainly lymphocytes. Lymphopholicullar proliferations occurred predominantly in perivascular and peribronchial sheaths. Hyperplasia of smooth muscle in the walls of terminal bronchioles and alveolar ducts was detected. The most characteristic feature was hyperplasia of alveolar type II epithelial cells, lining the alveolar septa. Another characteristic finding was presence of an amorphous, intensely eosinophilic material in most alveolar spaces. In the mammary glands, focal to multifocal interstitial infiltration with mononuclear cells and fibrosis of the alveolar parenchyma and lymphoid proliferation around lactiferous ducts were detected.

Phylogenetic analysis revealed that all isolates from sheep, as well as the isolates from the goats in the mixed sheep and goat farm were clustered into the A2 and A4 subtypes. In contrast, all isolates from goats in the exclusively goat herd were clustered into the B1 subtype.

Discussion and conclusions

Greek SRLVs from flocks with sheep (whether from sheep or from goats in a mixed sheep and goat farm) were closer to sheep viruses. It is noteworthy that the lentivirus isolated from the goats in the mixed sheep and goat farm aligned within the cluster of the ovine isolates of the virus. This is the first report of a group A2 isolate obtained from goats with clinical pneumonia. This provides strong evidence for natural transmission of the virus from sheep to goats. Furthermore, the strain was able to cause clinical pneumonia in affected goats. Interspecies transmission of SRLVs can occur in the field, as suggested by the detection of some subtypes in both sheep and goats (2). Recently, direct evidence was found proving that, in mixed farms of sheep and goats, the subtypes A4 and B1 may indeed jump the species barrier (3, 5). These findings probably refute the current theory that possibly some SRLVs can be better adapted to sheep, whilst other ones to goats.

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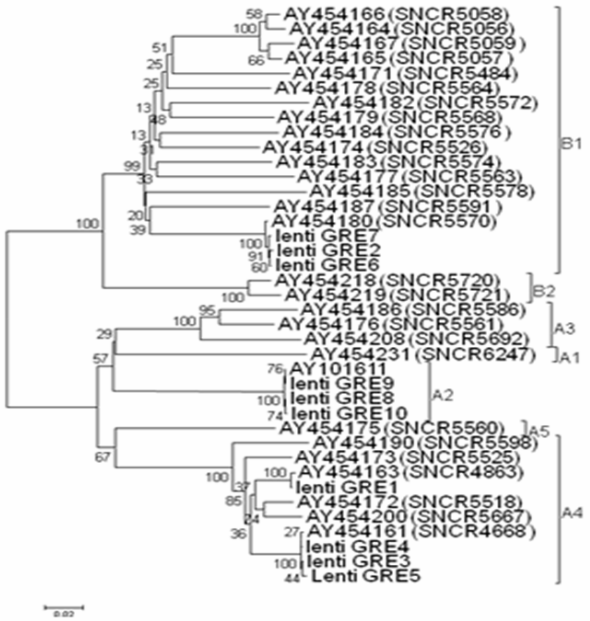

Figure 1. Neighbor-joining tree presenting the relationship of lentiviruses.

FIRST DESCRIPTION OF SWINE TORQUE TENO VIRUS (TTV) AND DETECTION OF A NEW GENOGROUP IN HUNGARY

Mária TAKÁCS[1], Ágnes DENCS[1], Csenge CSISZÁR[1], Andrea HETTMANN[1], Erzsébet RUSVAI[1], Katalin N. SZOMOR[1], Vilmos PÁLFI[2], Béla NAGY[3]

[1]*Division of Virology, National Center for Epidemiology, Budapest;*
[2]*Central Agricultural Office Veterinary Diagnostic Directorate (former Central Veterinary Institute), Budapest;*
[3]*Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary*

Keywords: genogroups, swine, TT virus

Introduction and objectives
Torque teno virus (TTV) was discovered in 1997 in Japan, in the serum of a human patient with posttransfusion hepatitis of unknown etiology (2). Torque teno virus (TTV) belongs to the genus Anellovirus, which contains small, non-enveloped viruses with a single-stranded, circular DNA genome. The TT virus has a genome size between 2.1 and 3.8 kb with negative polarity. TTV has a wide range of sequence divergence. Human TTV is distributed worldwide, but it has not been linked to any specific disease so far. TTV infects not only the liver but also many other organs. Human individuals may carry several distinct genotypes. TTV has also been detected in farm animals (pigs, chicken, cows and sheep) and in dogs and cats. TTV is highly prevalent in wild boar and it has been found in non-human primates and tupaias (4). To date, the full sequence of five TTVs identified in swine have been published. Swine TTV seems to be common in European pig herds. So far, two TTV genogroups have been described in swine: genogroup 1, AB076001 (4), and genogroup 2 AY823990 (1).

Materials and methods
Serum samples of 82 adult swine from 13 piggeries and 44 weaned pigs from one large herd were tested by PCR for the presence of TTV DNA. Different organs of the weaned piglets was also tested. DNA purification of swine serum samples was performed with phenol/chloroform deproteinization after proteinase K digestion, followed by precipitation by isopropanol using standard methods. Serum samples of weaned pigs were processed with a Roche High Pure Viral Nucleic Acid Kit according to the manufacturer's protocol. Liver and intestine samples were pretreated as follows: the tissue was rubbed with sterile sand and homogenized in 1,5 ml cold PBS pH 7,2. The tissue homogenate was centrifuged and the supernatant was processed immediately. Primers specific for swine TTV were used to detect TT virus. 5 µl of the product was checked on an agarose gel. PCR products were cloned and sequenced on a MegaBACE flexible 1000 capillary sequencer using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences Corp.). DNA sequences were aligned using the Multalin software.

Results
25 of 82 (30%) swine sera proved to be positive for TTV. We received at least one positive sample from almost every piggery. 32 of the 44 weaned piglets sera (73%) were found to be positive for TT virus DNA. We also tested 16 liver and 22 intestine samples of these 44 weaned piglets, from which 5 of the 16 liver samples and 4 of the 22 intestine samples proved to be positive. Both the liver and the intestine were positive in case of 2 piglets. Two piglets' liver were found to be positive without the positivity of serum or intestine and 8 piglets had viraemia without positivity in other organs tested. No specific clinical signs were assigned to these pigs. All the samples (liver, intestine and serum) were positive only from one piglet. PCR products obtained from the organs of this piglet were cloned, and the nucleotide sequence of several clones was determined (GenBank Accession number: FM200045-52) and aligned with two previously published swine TTV isolates. A phylogenetic tree was constructed. Sequences originated from sera and intestine of this weaned pig were phylogenetically closest to swine TTV isolate AY823990 (1). However, clones derived from the liver represented a remarkably different genotype. They were only distantly (80%) related to isolate AB076001 (4), indicating the presence of a new genogroup.

Discussion and conclusions
Based on these studies, the countrywide presence of TTV has been demonstrated in Hungarian piggeries. The prevalence of TT virus in weaned pigs was significantly higher than in adult swine. TT virus was present in different organs of weaned piglets without clinical symptoms. The sequence of viruses derived from sera and intestine were very similar, but remarkably different TT virus sequences were identified in the liver of one weaned pig. As it had been published earlier for humans (3,5) mixed infection could be found in weaned pigs, as well. Like in humans distinct virus subtypes were detected in different tissues in a piglet. Here we sequenced a 500 bp long segment of the ORF1. Our results show that significantly distinct sequences may be present in different tissues, suggesting that certain virus genotypes might be better adapted to particular cell or tissue types. Furthermore, the sequences we found in the liver, differ remarkable from the known genogroups, and may represent a new genogroup.

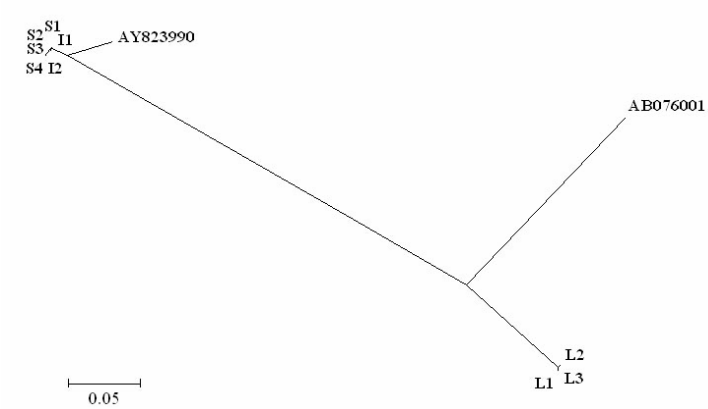

Figure 1. Phylogenetic tree of viral sequences derived from different organs of weaned pig. Organs : L = liver,S = serum,I = Intestine

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CHARACTERISATION OF THE MURINE ADENOVIRUS 2 GENOME AND PARTIAL SEQUENCES FROM SIMILAR RODENT ADENOVIRUSES

Márton VIDOVSZKY[1], Sandra RAMELLI[2], Willy DECURTINS[2], Justyna RUMINSKA[2], Andor DOSZPOLY[1], Gabriella SKODA[1], Máté JÁNOSKA[1], Balázs HARRACH[1], Urs GREBER[3], Silvio HEMMI[2]

[1]Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary; [2]Institute of Molecular Biology, University of Zürich, Switzerland; [3]Institute of Zoology, University of Zürich, Switzerland;

Keywords: agouti, guinea pig, hamster, mouse, murine adenovirus 2

Introduction and Objectives

Presently, the family *Adenoviridae* consists of four genera approved by the ICTV (International Committee on Taxonomy of Viruses) [1]. Adenoviruses (AdVs) are hypothesized to have co-evolved with their vertebrate hosts [2]. One host species can harbour several AdVs, some of which might even have coevolved originally with a different animal species. Such "host-switching" AdVs seemingly may cause more severe diseases. Our objective is the study of the phylogeny of rodent AdVs.

Materials and methods

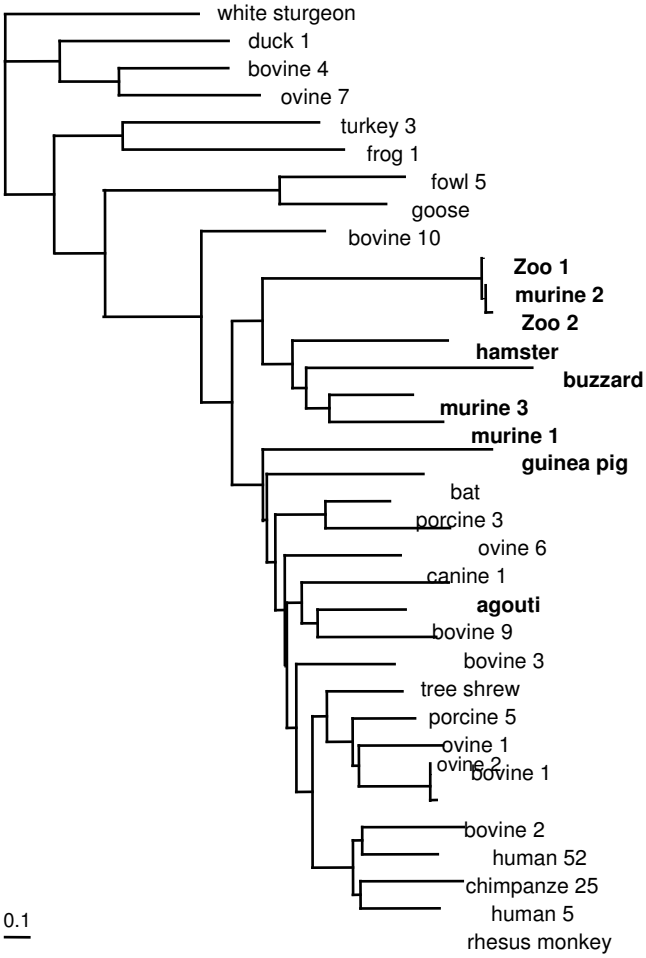
Murine adenovirus 2 (MAdV-2) strain K87 was propagated in tissue-culture, its DNA was extracted and the full genome was sequenced by common molecular methods. The genome was analysed by homology search and by comparing it to earlier sequenced and annotated AdV genomes. Phylogenetic calculations were performed by the Phylip program package. Parallel, a search for novel adenoviruses applied the highly effective, pan adenovirus PCR method, published earlier [3], to detect AdVs in random tissue or faeces samples.

Results

The full genome of MAdV-2 strain K87 was sequenced and found to be 35'203 bp long with a G+C content of 63.35%. It is considerably longer than the genome of MAdV-1 and MAdV-3 the very recently isolated and sequenced novel AdV isolated from Eurasian field mouse (*Apodemus agrarius*) [4] that have a length of 30'944 and 30'570 bp, respectively. The comparative analysis revealed that the size difference is rather the result of longer, than higher number of, genes. On the phylogenetic tree, MAdV-1, -2, and -3 are monophyletic, and seemingly are more ancient than other studied mastadenovirus. From these murine AdVs, MAdV-1 and -2 are much more similar to each other than to MAdV-2. The genome organization of the three MAdVs is similar with slight discrepancies. The first gene of the E3 region (12.5K) is present in MAdV-2 but is missing from MAdV-1 and -3. ORFA, ORFB, ORFC, situated at the right-hand end of the MAdV-1 genome seem to be missing from MAdV-2, although ORFA and ORFC are present in MAdV-3. A pan-adenovirus PCR found further AdVs similar to but not identical with MAdV-2.

Discussion and conclusions

The three MAdV genomes have characteristic differences in the E3 and E4 regions. The E3 12.5K gene is present only in MAdV-2. This gene codes a non-essential protein which is present in most of the mammal adenoviruses [5] but its function is not known yet. From the five E4 genes of MAdV-1 (ORFA to -E) ORFA, -B, and -C could not been found in MAdV-2, while MAdV-3 seem to lack only ORFB. The function of these three putative proteins is not known either. The genetic distance between MAdV-1 (or MAdV-3) and MAdV-2 well exceeds 10%, thus it is clear that a separate species (with a proposed name of Murine adenovirus B) should be officially established for MAdV-2. While analysing environmental samples by the pan-adenovirus PCR (supposed to detect all AdVs), viruses very similar to MAdV-2 were found. These samples originated from a zoo, and we speculated that mice living in the area could be the source of this contamination. With the same primers, we found a less similar mastadenovirus from common buzzard (*Buteo buteo*). As this is the only time of recovering a mastadenovirus from avian (or any non-mammalian) sample, we suppose that the amplified viral DNA originates from the prey of the bird. This AdV has a phylogenetic place between MAdV-1 and -2. Thus it seems to be logical to hypothesize that the prey was some kind of rodent (a frequent prey of common buzzard). PCR identified a novel adenovirus also from hamster (*Cricetus cricetus*). It seemed to be monophyletic with the earlier mentioned rodent AdVs. These phylogenetic data suggest a common evolutionary root for all of these Muroidea AdVs, i.e., a new proof for their co-evolution. We could amplify AdV sequence also from rodents that are phylogenetically more distant: agouti and guinea pig (Hystricognathi). They appear with a greater distance on the phylogenetic tree. As we found several AdVs almost identical with MAdV-2, while MAdV-1 and -3 seem to be more unique and more pathogenic, one may hypothesize that MAdV-2 is the "real" adenovirus of mice (that coevolved with this rodent species), whereas MAdV-1 and -3 may have been acquired by house mouse and Eurasian field mouse from some other rodent species.



Acknowledgements

Thanks are due to Klaus Überla for providing the sequence of MAdV-3 before publication. Thank to Susan Compton and Jim Pipas for help in getting MAdV-2 DNA. This study was partially financed by grant OTKA K72484.

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AN INFECTIOUS CLONE OF WEST NILE VIRUS IS-98-ST1

Céline BAHUON[1], Sylvie LECOLLINET[1], Eva MERTENS[2], Stéphan ZIENTARA[1], Philippe DESPRES[2]

[1]UMR1161 Virologie, AFSSA, INRA, ENVA, 23 avenue du général de Gaulle, 94703 MAISONS-ALFORT, France;
[2]Unité Interactions Moléculaires Flavivirus-Hôte, Institut Pasteur, 25-28 rue du Docteur Roux, 75724 PARIS cedex 15, France

Keywords : West Nile virus, neurovirulence, molecular determinants, infectious clone

Introduction and Objectives

West Nile virus (WNV) is a neurotropic flavivirus transmitted by mosquitoes. Wild birds are the amplifying reservoir hosts. Equids and humans are incidental hosts particularly sensitive to the infection. In humans, the infection can lead to symptoms (paralysis, cognitive disorders) caused by neuronal injury (Guarner et al., 2004), consequence of host inflammatory reaction and/or direct action of the virus on neurons.

Understanding the molecular mechanisms of virulence for neuronal cells is of major importance for public health organisms to detect and react to a new virulent strain. Cell death by apoptosis (Samuel et al.;2007), and persistance of the virus (Hunsperger et al., 2005) have both been observed in mouse neuronal cell primary cultures.

Pathogenicity for human neuronal cells and also molecular mechanisms involved in neuronal injury in humans are not well known.

We aim at studying the impact of WNV infection on human neurons in the absence of host inflammatory reaction (with primary cultures or neuroblastoms) and at deciphering the molecular basis of virulence in this model.

For this latter objective, we are designing an infectious clone of the IS-98-ST1 WNV strain, which is a particularly virulent WNV strain from Israel (Lucas et al., 2004). Chimeric constructions and directed mutations will be used to identify the genes and amino acids involved in the virulence of the virus.

Materials and methods

Flaviviruses infectious clones are known to be unstable in *Escherichia coli* (Liu *et al*., 2003).We chose to subclone the viral genome in pBR322, a low-copy-number vector, in order to avoid potential recombinations and mutations. A plasmid coding for the 5' and 3' non coding regions was manufactured by Genecust. Sp6 promoter and HDV ribozyme sequence were inserted respectively before the 5' end and after the 3' end of the viral genome. IS-98-ST1 genomic RNA was reverse-transcribed and amplified into four fragments: fragment one coding for structural proteins, fragment two coding for NS1, NS2a/b, fragment three coding for NS3, NS4a, and fragment four coding for NS4b and NS5. The chosen strategy was to split the full-length cDNA into two pieces in order to facilitate latter incorporation of mutations.

Results

A first plasmid with fragments one and two is currently under construction, and a second plasmid containing fragments three, four and a fraction of fragment two has been obtained. RNA will then be transcribed from the *in vitro* ligated or recombined DNA template.

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COMPARISON OF THE LONG FIBER OF SEROTYPE 1 FOWL ADENOVIRUS ISOLATES FROM CHICKENS WITH GIZZARD EROSIONS

Ana BASIC, Evelyn SCHULZ and Michael HESS

Clinic for Avian, Reptile and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria

Keywords: fowl adenovirus, long fiber, gizzard erosions

Introduction and Objectives

Fowl adenoviruses (FAdVs) belong to the family *Adenoviridae* and genus *Aviadenovirus*. They are endemic worldwide and can be isolated from both healthy and sick birds. A pathogenic role of FAdV serotype 1 (FAdV1) in gizzard erosions was shown (Okuda et al., 2001). However, Ote strain failed to induce gizzard erosions and CELO strain is responsible just for mild respiratory pathologies in birds (both strains belong to FAdV1). Therefore, it was postulated that variably pathogenic FAdV1 strains exist in Japan: some capable of causing gizzard erosions ('pathogenic') and the others, which are incapable of causing these lesions ('apathogenic') (Okuda et al., 2006). Recently, gizzard erosions associated with FAdV have been found in chickens in Europe. Contrary to most adenoviruses, FAdV1 has two fibers of different lengths (Hess et al., 1995). The distal parts (or heads) of these fibers are involved in cellular receptor(s) binding. Moreover, it was indicated that fibers of FAdV play an important role in infectivity and pathogenicity of FAdV (Pallister et al., 1996). In addition, it was suggested that PCR-RFLP analyses of long fiber genes could be used to distinguish between 'pathogenic' and 'apathogenic' FAVd1 strains in regard to gizzard erosions (Okuda et al., 2006). The objective of this study was to analyze the nucleotide and amino acid sequences of the long fiber of several isolates from broiler chickens with gizzard erosions and compare them to those of 'apathogenic' FAdV1 strains: Ote and CELO.

Materials and methods

A total of 18 virus isolates from Europe were used in this study. All samples were obtained from birds with gizzard erosions. Viral DNA was extracted from the supernatant of infected cell culture fluids. Using PCR for the loop 1 region of hexon gene, the presence of FAdV was determined and isolates were identified as FAdV1 by sequencing of PCR products. Oligonucleotide primers for the amplification of DNA, corresponding to the entire long fiber gene were designed on the basis of the published sequence data of the CELO strain (Chiocca et al., 1996). PCR products of approximately 2382 bp were visualized by agarose gel electrophoresis and further analyzed by RFLP analysis (using *Hinf*I) and sequencing. For comparison, 'apathogenic' FAdV1 strains: Ote and CELO were used. Long fiber gene sequence of completely sequenced CELO strain was used in sequence analysis (Chiocca et al., 1996). In addition, predicted amino acid sequences of long fiber of isolates from birds with gizzard erosions and 'apathogenic' isolates were compared.

Results

PCR products of entire long fiber gene of studied isolates were 2382 bp long, as expected, and that of Ote and CELO strains were the same size. The patterns from the PCR-RFLP analysis of some isolates are shown in Figure 1. Surprisingly, the RFLP patterns of all studied isolates were the same and they were the same as that of 'apathogenic' FAdV1 strains: Ote and CELO. No isolate had the 'pathogenic' type restriction pattern or 'mixed' restriction pattern (Okuda et al., 2006). Sequence analyses of the long fiber gene of FAdV1 isolates and prediction of *Hinf*I recognition sites confirmed the results obtained by PCR-RFLP analysis. Several nucleotide differences between these isolates and the 'apathogenic' FAdV1 strains were noticed in the long fiber gene. However, all mutations in the head domain of the long fiber gene between studied isolates and the CELO strain were silent mutations. In head domain of the long fiber of Ote strain, 1 non-silent mutation (A→S) was determined.

Discussion and conclusions

The factors that determine the pathogenicity of FAdV are still not clear. In the study reported here, PCR-RFLP analysis of the long fiber gene (using *Hinf*I) was used in attempt to segregate FAdV1 isolates into 2 groups that differed in their ability to induce gizzard erosions as suggested earlier (Okuda et al., 2006). However, the restriction patterns of all FAdV1 isolates from birds with gizzard erosions were the same as that of Ote and CELO strains that are shown not to induce gizzard erosions. In addition, this result was confirmed by sequence analysis. In conclusion, the usefulness of PCR-RFLP analysis of the long fiber gene of FAdV1 isolates in distinguishing between those that induce gizzard erosions and those that do not remains questionable. Moreover, nucleotide and amino acid sequence analyses of the long fiber gene indicate that long fiber does not seem to determine the pathogenicity of FAdV1 isolates at least in regard to gizzard erosions. Further studies of the long and short fiber are needed to investigate their role in the pathogenicity of FAdV1. In addition, in order to estimate the influence of viral genetics on pathogenicity, experimental infections with different FAdV1 isolates would be necessary keeping in mind that FAdV1 is often vertically transmitted and can be isolated from healthy birds as well.

Acknowledgements

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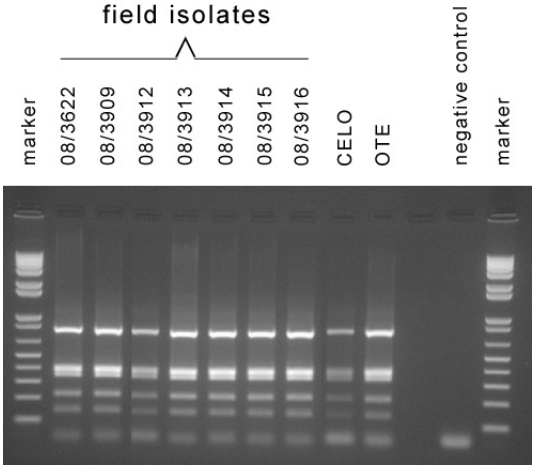

Figure 1: Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) patterns of fowl adenovirus serotype 1 (FAdV1) isolates from birds with gizzard erosions and CELO and Ote strains.

COMPARATIVE IMMUNOHISTOCHEMICAL STUDIES ON THE TISSUE DISTRIBUTION AND PERSISTENCY OF THREE VARIANTS OF CLASSICAL SWINE FEVER VIRUS IN EXPERIMENTALLY INFECTED PIGS: A RECENT ISOLATE OF WILD BOAR ORIGIN, A NEWLY DEVELOPED MODIFIED LIVE MARKER VACCINE AND THE CONVENTIONAL "C" VACCINE STRAIN

Katinka BELÁK[1,2], Marylène TIGNON[3], Gábor KULCSÁR[4], Tímea BARNA[4], Katalin FÁBIÁN[4], Réka LÉVAI[4], Attila FARSANG[4], Frank KOENEN[5], Sándor BELÁK[1]

[1]*Joint Research and Development Division, Department of Virology, Immunobiology and Parasitology, National Veterinary Institute and Swedish University of Agricultural Sciences, S-751 89, Uppsala, Sweden;*

[2]*Department of Pathology and Wildlife Diseases, National Veterinary Institute, S-751 89, Uppsala, Sweden;*

[3]*Veterinary and Agrochemical Research Centre (VAR), Operational Directorate of Virology, Groeselenberg 99, B-1180 Brussels, Belgium*

[4]*Central Agricultural Office, Directorate for Veterinary Medicinal Products (DVMP), Szállás utca 8, H-1107 Budapest, Hungary*

[5]*Veterinary and Agrochemical Research Centre (VAR), Operational Directorate of Interaction and Surveillance, Groeselenberg 99, B-1180 Brussels, Belgium*

Keywords: CSFV, CP7_E2alf, immunohistochemistry

Introduction and Objective

The virus distribution and the invasive characteristic of three virulence variants of classical swine fever virus (CSFV) were studied in various organs in three groups of experimentally infected pigs by immunohistochemistry (IHC) (1): a *Rostock* type CSFV strain of wild boar origin, a newly developed live marker vaccine, as well as the reference "C" vaccine strain.

By comparing the spread of these virulence variants in 116 animals, this study is one of the most comprehensive investigations performed so far to examine the tissue distribution of CSFV in the natural host, in order to obtain data of comparative virus distribution. These observations will contribute to a better understanding the viral pathogenesis of the newly developed live marker vaccine, CP7_E2alf (2), and to the introduction of more effective measures to control classical swine fever (CSF).

Materials and methods

In experiment 1, twenty-eight pigs were oronasally infected with $10^{3.8}$ $TCID_{50}$ (2 ml) of the '11722-WIL' strain of CSFV (*Rostock* type). In experiment 2, forty-four pigs were vaccinated with $10^{4.5}TCID_{50}$ /ml (2 ml) of the newly developed, live marker, CP7_E2alf vaccine. In experiment 3, forty-four pigs received 2 ml of $10^{4.5}TCID_{50}$/ml of the conventional "C" strain vaccine.

In experiment 1, two non-infected animals were slaughtered on the day of infection as negative controls; the infected animals on a daily basis during the first eight days post infection (dpi); on a two-day basis from 10 until 14 dpi; and finally at 17 dpi. Remaining animals either died spontaneously or were humanely killed for ethical reasons when moribund at 20, 23 and 24 dpi.

In experiments 2 and 3 on day 0, all animals, except the two negative controls, were oronasally vaccinated. The two negative controls were slaughtered on the vaccination day. Two vaccinated animals were randomly slaughtered on a daily basis between 0 and 8 days post vaccination (dpv), at 13 and 18 dpv, and then weekly from 21 to 98 dpv.

After euthanasia or death, necropsies were performed and gross lesions were recorded. Tissue samples of tonsils, spleen, thymus, ileocoecal, mesenteric and submandibular lymph nodes, ileum, kidneys, lungs, heart muscle, cerebrum, cerebellum and striated muscle (M. longissimus dorsi and M. quadriceps) were collected from all animals.

For immunohistochemical examinations, the collected tissue samples were fixed in 4% paraformaldehyde, embedded in paraffin according to routine histological procedures and sectioned at the thickness of 5 µm.

Commercially available monoclonal antibodies "WH 303", specific to CSFV glycoprotein E2, was applied (Veterinary Laboratories Agency-Weybridge, New Haw, Addlestone, UK) by a two-step peroxidase method, using the DakoEnVision +HP mouse Kit (Dakopatts, Glosstrup, Denmark).

Results

Experiment 1

The *Rostock type* virus antigen was demonstrated by IHC in the various tissue samples of the infected pigs. The virus first was detected at 1 dpi in the tonsil in one pig. From 2 dpi the virus was consistently demonstrated from the tonsil samples in all of the animals until the end of the experiment at dpi 24. From 2 to 24 dpi, the *Rostock type* virus was detected in 21 pigs in the tissue samples from spleen and submandibular lymph node. From 17 to 23 dpi the virus antigen was detected in three pigs in the tissue samples from kidney respectively from 5 to 20 dpi in seven animals in ileum samples. The virus was not detected in samples from brain tissues and striated muscles.

Experiment 2

The CP7_E2alf virus antigen was first detected at 2 dpi in the tonsil of one pig. Between 2 and 8 dpi the virus was revealed in five animals: in four animals in the tonsil and in the fifth one in the submandibular lymph node. The virus was not detectable between 13 and 77 dpi. At 77 dpi it was demonstrated in the tonsils of both euthanized pigs and in addition in the spleen and the three lymph node samples in one of them. At the last day of the experiment, the marker vaccine candidate could be demonstrated again in the tonsils and spleen.

Experiment 3

"C" strain antigen was demonstrated by IHC in the various tissue samples of 28 pigs. The virus first was detected at 1 dpi in the tonsil in one of the two euthanized pigs and then at 3 dpi in tonsil and surprisingly in ileocoecal lymph node and ileum. From 1 to 91 dpi the vaccine strain was consistently demonstrated from the tonsil samples in 20 pigs. From 8 to 91 dpi the "C-strain" was detected in nine pigs in the tissue samples from kidney. The virus was not detected in samples from thymus, lungs, brain tissues and striated muscles.

Discussion and conclusions

By the means of IHC, the wild type CSF virus antigen was detected in the tonsils from dpi 1 in all the pigs but one. Additionally the virus antigen was revealed in the spleen in most of the animals. Positive immunohistochemical staining was seen in lungs, kidneys and ileum from about half / final part of the experiment.

In experiment 2, the live marker vaccine candidate, CP7_E2alf could be detected exclusively in the lymphoid organs in a few cells totally in nine animals from dpi 2 to 13 and then at dpi 77 and 98.

In experiment 3, the "C" strain vaccine antigen was demonstrated in the tonsils, lymph nodes, spleen, kidneys and ileum in half of the all animals (in 28 pigs). The virus antigen was constantly demonstrated in the tonsils of 20 pigs between dpi 1 and 91.

In conclusion, all the three viruses, the wild virus, CP7_E2alf, and the "C-strain" were replicating in the tonsils.

This method provides useful means of early virus detection, indicates the localization of the virus spread and the invasion capacity in various tissues, supporting the determination of the pathogenicity levels of the different virulence variants of CSFV, including vaccine viruses. IHC is a useful assay for studying duration of the viral replication process. Thus, it is a practical tool to study and compare the vaccine candidates.

Acknowledgements

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AVIAN INFLUENZA VIRUS (AIV) PATHOGENESIS IN A FERRET MODEL: H5N1 CLADE 2.2 (A/TURKEY/TURKEY/1/05)

Sharon M. BROOKES[1], Fanny GARCON[1], Dianne MAJOR[2], Lorenzo RAPISARDA[1], Alejandro NUNEZ[1], John WOOD[2], Ian H. BROWN[1]

[1] Veterinary Laboratories Agency – Weybridge, Department of Virology, Addlestone, United Kingdom
[2]National Institute for Biological Standards and Control, Department of Virology, Potters Bar, United Kingdom

Keywords: Avian Influenza, Pathogenesis, Ferret Model.

Introduction and Objectives

As ferrets are naturally susceptible to infection with human Influenza viruses due to the similarity in virus receptor and the clinical disease representation, they represent a good model to study influenza pathogenesis and immunity studies (1).

Previous studies have generally involved the use of highly pathogenic AIV from human cases. The objective of our study was to investigate the pathogenesis of a naïve avian isolate, A/turkey/Turkey/1/05 H5N1 in a ferret model.

Materials and Methods

Pre-bleeds plus oral and anal swabs were carried out to eliminate previous exposure to influenza A by MultiS-Screen AI ELISA, Haemagglutination Inhibition (HI) and H5 Real time RT-PCR.

The outbred ferrets were 7-8 months old with an average weight of 1-2kgs. The group sizes were either 3 or 5. Each group represented the three different phenotypes available (albino, polecat and sandy).

Ferrets were infected intranasally with stock virus: A/turkey/Turkey/1/05, at one of three doses:

I) a 10^7 EID_{50} dose of egg passage 3 (EP3),

II) a 10^6 EID_{50} dose of previously plaque purified virus (EP2/MDCKpp/EP1) or

III) a 10^4 EID_{50} dose of EP2.

The inoculum was delivered as a total dose of 0.4ml, 0.2 ml per nostril.

Ferrets were observed daily. Rectal and oral swabs plus nasal washes were collected every other day and processed for H5 RRT-PCR. Post-mortems were carried out at dpi 4, 6 and 13. Tissues were processed for H5 RRT-PCR, virus isolation on cells and histopathology.

Results

Viral infection induced substantial clinical signs including pyrexia (>39°C) from dpi 2-7, substantial weight lost (up to 20% total body weight), lethargy, hypersalivation, respiratory and neurological distress. Disease recovery was therefore reduced; EP2 and EP3 viruses induced 66 and 40% mortality respectively. The plaque purified virus induced only mild disease with raised temperatures from dpi 2-4 and no significant weight loss followed by full recovery.

Virus shedding was detected via the nasal cavity of all infected animal by H5 real-time RT-PCR. Infection with EP viruses induced a peak of virus shedding from dpi 1-9 whereas with the plaque purified virus shedding was reduced during dpi 1-7. No virus shedding was observed in oral and rectal cavities.

Homologous antibody levels against H5N1 were detected from dpi 13 in infected animal.

Presence of influenza A virus was detected by immunohistochemistry and virus isolation from the nasal cavities, lung and brain. Clear consolidated lung lesions were visible from dpi 4 and 6 indicative of influenza infection.

Discussion and Conclusion

A naive AIV (A/turkey/Turkey/1/05) induced typical influenza A spectrum clinical disease in the ferret model. The passage history of the virus is the main factor affecting disease pathogenesis of H5N1 viruses in this model. The virulence of the virus is substantially reduced when the egg passage number increases and by plaque purification on cells.

As this naive AIV is able to induce lethal disease in ferret model, it could therefore continue to represent a substantial threat to human health.

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CHARACTERIZATION OF IN VITRO PARAMETERS CORRELATING WITH VIRULENCE OF CLASSICAL SWINE FEVER VIRUS IN PIGS

Rita EYMANN-HAENI[1], Artur SUMMERFIELD[1], Sandra BLOME[2], Irene GREISER-WILKE[2], Nicolas RUGGLI[1]

[1]Research Department, Institute of Virology and Immunoprophylaxis, CH-3147 Mittelhäusern, Switzerland;
[2]Institute of Virology, EU Reference Laboratory for Classical Swine Fever, Department of Infectious Diseases, University of Veterinary Medicine, D-30559 Hannover, Germany

Keywords: CSFV, virulence, in vitro assay, focus size, replication

Introduction and Objectives
Classical Swine Fever Virus (CSFV) is a highly contagious disease of pigs. Wild boar, the natural reservoir for CSFV, represents the main source for transmission of CSFV to domestic pigs. Importantly, different CSFV isolates from wild boar vary in their degree of virulence for pigs. To allow appropriate surveillance and control measures to be implemented, it is important to assess the virulence of a wild boar CSFV isolate for domestic pigs. Little is known on the CSFV determinants of virulence. It has been shown that the viral envelope glycoproteins E^{rns}, E1 and E2 play an important role in virulence [1-3]. Currently, virulence of a CSFV strain can only be determined by experimental infection of pigs. In vitro parameters for estimation of virulence would contribute to reduce the number of animal experiments. Therefore, the present study is aimed at identifying in vitro parameters that correlate with virulence. To this end we analysed virological and immunological parameters on an aortic endothelial cell line and on primary porcine cells, using a collection of CSFV isolates of defined virulence.

Materials and methods
Sixteen CSFV isolates of defined virulence were amplified from animal material by 3 serial passages on the porcine PEDSV-15 cell line [4]. This cell line does not select for heparan sulfate-adapted mutants [5]. The collection of 16 isolates consists of four highly virulent, eight moderately virulent, two low virulent and two avirulent CSFV strains. Six isolates are from the EU Reference Laboratory for Classical Swine Fever, Department of Infectious Diseases, University of Veterinary Medicine, Hannover, Germany. The other isolates were characterized in pigs at the Institute of Virology and Immunoprophylaxis, Mittelhäusern, Switzerland.
For virus focus size analysis, PEDSV-15 cells were infected at low multiplicity of infection (MOI). After virus adhesion, cells were washed and incubated under a semi-solid agarose medium overlay. To determine the focus size, cells were fixed after three days and stained for viral glycoprotein E2 expression using standard immunoperoxidase technique.
To analyse the kinetics of virus replication, PEDSV-15 cells were infected at a low MOI. Accumulation of de novo synthesized virus was quantified by titration of whole cell extracts on PEDSV-15 cells at early time points post-infection.

The infection of cells of the porcine immune system was evaluated in porcine monocyte-derived macrophages (MDM) and plasmacytoid dendritic cells (pDC). The cells were prepared from blood of specific pathogen-free pigs by standard procedures. Virus infection was analysed by flow cytometry using antibodies against the viral NS3 protein and against cell surface markers.

Results
In vivo, highly virulent CSFV strains result in earlier viremia and in higher viral load than low virulent strains. Therefore we hypothesized that the efficiency of CSFV spread in an appropriate cell culture system may be related to the virulence of a particular isolate in vivo. To this end we assessed three different parameters: (i) focus formation, (ii) kinetics of virus replication and (iii) efficiency of infection of ex vivo target cells of CSFV.

First, we analysed the focus formation in PEDSV-15 cells under a semi-solid medium overlay. We show that highly virulent strains such as Eystrup, Koslov and Brescia form significantly larger foci than moderately virulent or avirulent strains. It is well established that CSFV prevents interferon-α/β induction in most cell systems. Therefore we assume that focus formation is not influenced by cellular antiviral defence, but rather reflects the efficiency and rapidity of virus replication and cell-to-cell spreading.

Second, we monitored the kinetics of the viral life cycle in PEDSV-15 cells. To this end we infected cells with low MOI and determined the time at which progeny virus can first be detected. The kinetics analyses revealed that highly virulent strains can be detected earlier after infection than low virulent strains, indicating that the speed of de novo virus synthesis in vitro may correlate with virulence in vivo.

Third, we investigated the interaction of CSFV with porcine cells representing the natural targets of the virus, i.e. MDM and pDC. Early after low MOI infection of pDC, we repeatedly observed a higher percentage of infected cells with the highly virulent CSFV strains than with the moderately virulent and avirulent strains. Also, the fluorescence intensity that is proportional to the amount of viral antigen expressed in the cells was higher with highly virulent than with avirulent strains. Similar results were obtained with infected MDM, in particular with interferon-γ pre-stimulated MDM.

Discussion and conclusions
A set of 16 CSFV isolates of different virulence was prepared on the porcine PEDSV-15 cell line [4] that we found not to select for heparan sulfate-adapted mutants [5]. This allowed a systematic comparison of defined isolates with respect to in vitro parameters that may correlate with virulence in domestic pigs. Our data provides strong evidence that the efficiency of CSFV replication in an appropriate cell culture system represents the fitness of the virus and is indeed related to the virulence of a particular isolate in vivo. These analyses proved more informative than detailed dissection of the different steps of the viral life cycle. Considering that virulence in vivo is a complex feature influenced by viral and host factors, we postulated that the prediction of virulence cannot rely on a single in vitro assay, which is suggested by our data. Therefore, we propose an approach based on a combination of several in vitro parameters to predict the virulence of CSFV.

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MODULATION OF CYTOKINES AND INTERFERON TYPE 1 PATHWAY BY AFRICAN SWINE FEVER VIRUS OF DIFFERENT VIRULENCE, ASFV/L60 AND ASFV/NH/P68

Solange GIL[1], Nuno SEPÚLVEDA[2], Emmanuel ALBINA[3], Alexandre LEITÃO[4], Carlos MARTINS[1]

[1]*Laboratório de Doenças Infecciosas, CIISA, Faculdade de Medicina Veterinária, Technical University of Lisbon (TULisbon), Av. da Universidade Técnica, 1300 - 477 Lisboa, Portugal;*
[2]*Instituto Gulbenkian de Ciência, Oeiras Portugal;Center of Statistics and Applications, University of Lisbon, Lisboa; Escola Superior de Saúde Egas Moniz and Instituto Superior de Ciências da Saúde Egas Moniz, Monte da Caparica, Lisboa, Portugal;*
[3]*CIRAD, UMR Contrôle des Maladies, Montpellier, F-34398 France ;*
[4]*Instituto de Investigação Científica Tropical, CVZ, CIISA, Av. da Universidade Técnica, 1300 - 477 Lisboa, Portugal*

Keywords: ASFV, cytokines, IFN type I, macrophages

Introduction and Objectives

African swine fever virus (ASFV) the only member of the *Asfarviridae* (3) family infects all *Suidae* and different species of ticks (8). In the domestic pigs ASFV isolates of different virulence induce disease in domestic pigs ranging from fatal to chronic or inaparent forms (8). A relevant role for cellular and cellular based immune mechanisms in protection against ASFV is generally accepted because pigs may survive infection or challenge inoculations with different viral isolates in the absence of virus neutralization by antibodies. Among others the low virulent ASFV/NH/P68 (NHV) has been previously shown to induce ASFV specific CTL activity (6,7), enhancement of NK avtivity and protection against subsequent infection with the highly virulent ASFV/L60 (L60) (4). Independently of their virulence, all ASFV isolates replicate in porcine monocytes and macrophages *in vivo* and *in vitro* (5, 10), therefore it is expected that interactions between the virus and those cells determine relevant consequences on the viral pathogenesis and in the host immune responses. Cells of the macrophage lineage play among others an important role in the activation of pro-inflammatory and specific immune responses of the host. Macrophage-derived cytokines determine the development of inflammatory responses that are the first defense against infection and they influence the development of acquired immune responses towards Th1 or Th2 cell activation. Our previous studies pointed to the relevance of macrophage-derived cytokines on the pathogenesis of ASFV infection. The low virulent NHV induced clear increased levels of transcripts for TNFα, IL6, IL12 and IL15 at 6 hours post infection, in contrast to the effect of infection with L60 (1). More recently studies were developed to assess the expression of IFNα, TNFα, TGFβ and IL12p40 in macrophage cultures infected the above mentioned isolates. In this study we found a marked increased expression of IFNα in association with an enhancement of TNFα and IL12p40 in macrophages infected with NHV in comparison with L60 (2). At present and taking in consideration the marked increased expression of IFNα and the important role of IFNs type I in controlling virus infections, we have conducted studies by Real Time PCR aiming at identifying the impact of the ASFV infection in the expression IFN type I and its modulation through the expression of several factors involved in the IFN pathway, namely Jak1, Tyk2 and STAT1.

Materials and methods

Porcine blood-derived macrophage cultures from seven pig donors were obtained following culture of PBLs during 72 hours (2) and infected in vitro with either NHV or L60 at a multiplicity of 3 (MOI = 3). cDNA samples from individual macrophage cultures collected at 2, 4 and 6 h post-infection were used to quantify mRNA expression of IFNβ and of several factors involved in the IFN pathway, namely Jak1, Tyk2 and STAT1 by Real Time PCR.

Results

In our previous studies we found increased expression at mRNA levels and final products of IFNα, TNFα and IL12p40 in NHV infected porcine macrophages in comparison to the effect of infection with L60. In opposition, lower levels of mRNA expression of TGFβ were found in NHV infected macrophages.

Our current studies strongly suggest an increased expression at mRNA levels of IFNβ and of the factors involved in the IFN pathway namely Jak1, Tyk2 and STAT1, in NHV infection when compared to L60.

Discussion and conclusions

To date, little is known about the impact of interferon type I induction and regulation of ASFV infection. Type I IFNs have important biological functions, ranging from immune cell development and activation to tumour cell killing and, most importantly, inhibition of replication of many RNA and DNA virus (9). Overall our studies point to an increased expression of IFN type I in porcine macrophages infected *in vitro* with the low virulent NHV in parallel with increased expression of factors relevant for the Interferon pathway. Furthermore our studies reinforce and support the effect of the NHV infection by targeting innate immune responses relevant for pig survival against ASFV infection.

Ongoing studies are conducted to deeply clarify the modulation of the IFN pathway in NHV/L60 model of infection through the quantification of mRNA expression of STAT2, IRF9 and the measurement of their activation by Western Blot assays.

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DEVELOPMENT OF STRAND-SPECIFIC QUANTITATIVE RT-PCR ASSAY FOR BOVINE VIRAL DIARRHOEA VIRUS REPLICATION

S. Anna La Rocca[1], Rachelle Cook, Torstein Sandvik, Rebecca M. Strong

[1]Department of Virology, Veterinary Laboratories Agency, Addlestone, UK

Keywords: BVDV, replication, strand-specific RT-PCR

Introduction and Objectives

Bovine Viral Diarrhoea Virus 1 (BVDV-1), a pestivirus species within the Flaviviridae family, is a positive strand RNA virus. BVDV-1 is an important pathogen in cattle that can cause severe economic losses due to decreased fertility, abortions, diarrhoea and respiratory complications. Viral infections occur in acute and persistent forms. BVDV-1 replication occurs in membrane-associated complexes that are localised at the endoplasmic reticulum and generally resembles that of other positive strand RNA viruses. [2]. Replication starts with the synthesis of a negative strand copy of genomic RNA that then produces the double-strand replicative form. The negative strand RNA finally serves as a template for multiple rounds of positive strand RNA synthesis, leading to the asymmetric accumulation of approximately 10 positive strands for each negative strand. The presence of negative strand can therefore be used as marker for viral replication. Using a strand specific quantitative RT-PCR, the ratio of the negative to positive strand RNAs can be calculated and thus active viral replication identified. Quantitative negative strand RT-PCRs have been developed for other positive strand viruses including HCV [1] and FMDV [3]. To facilitate studies into viral replication during acute and persistent infection we have developed a quantitative, strand-specific RT-PCR for BVDV-1.

Materials and methods

The assay uses strand specific RT primers targeting 5'-UTR of the BVDV-1 genome. These primers include TAG sequences which allow subsequent amplification of the specific target molecule. Positive and negative strand control RNAs were generated by in vitro transcription (IVT) of plasmids encoding the 5'-UTR region of C24V strain of BVDV-1, orientated in opposite direction, using the SP6 promoter. The IVT RNA was purified by 3 cycles of treatment with DNAse and precipitation. RNAs were reverse transcribed using a specific TAG-forward or reverse primer at high temperature (62° C) with Thermoscript RT enzyme (Invitrogen). Reverse transcribed RNA was amplified by real time PCR using Brilliant-II PCR Mastermix (Stratagene) and a FAM labelled Taqman probe. The PCR step used a primer with the same sequence as the TAG on the RT primer that anneals 90 nucleotides downstream of the RT primers. RNA was harvested from Foetal Bovine Turbinate (FBT) cells infected with BVDV-1 strain C24V at varying time points up to 3 days post infection.

Results

Sensitivity of the assay for detection of both the negative and positive strands BVDV RNA was tested with 10-fold dilutions of the synthesized RNA (from 10^7 to 10^1 copies/µl). For both negative and positive strand 10^2 copies/µl could be detected. The assay also has a good efficiency and is linear from 10^2 to 10^8 copies/µl. Specificity has also been tested by preparing PCR reactions containing no RT enzyme, no template, and after addition of bovine cellular RNA or opposite strand RNA, and very little interference was detected only in the case of high amounts of opposite strand RNA.

The real time RT-PCR assay for the negative and positive strand BVDV RNA was found to be sensitive down to 10^2 copies/µl. There was no amplification in the negative controls. Initial results performing an in vitro time course analysis of C24V virus replication over 3 days have shown that within 24 hours there is a peak in the number of copies of the negative strand RNA followed by a peak of the number of copies of positive strand RNA, with a fall in the ratio of positive/negative strand RNA when the virus is undergoing very high levels of replication.

Discussion and conclusions

In the case of positive sense RNA viruses, an approach to evaluate levels of viral replication is to quantitate the ratio between negative and positive strand RNA in a given sample. The assay here developed applies the advantage that comes from the use of high temperature reverse transcriptase enzymes and tagged primers in the RT reaction for a greater specificity in the strand specific detection. The assay is sensitive enough to detect low copy numbers of both RNA strands. The assay has been evaluated using a time course experiment of BVDV infection over 72 hrs in cell culture, showing a high replication rate within 24 hrs. This method will prove useful in understanding the mechanism of RNA replication of BVDV and can also be applied to other members of the Flaviviridae family. This strand specific assay will be a very useful tool in determining the presence of replicating virus in acutely infected and persistently infected animals.

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CYTOKINE GENE EXPRESSION PROFILING AFTER INFECTION OF PORCINE CELLS WITH *CLASSICAL SWINE FEVER VIRUS* USING SAGE AND RT-QPCR

Anastasia LANGE[1], Irene GREISER-WILKE[1], Stefanie BENDFELDT[1], Laura C. MILLER[2], John D. NEILL[2]

[1] *Institute of Virology, Department of Infectious Diseases, University of Veterinary Medicine, 30559 Hannover, Germany*
[2] *National Animal Disease Center, ARS, USDA, 50010 Ames, Iowa, USA*

Keywords: *Classical swine fever virus*, gene expression, SAGE

Introduction and Objectives

Classical swine fever (CSF) is caused by the *Classical swine fever virus* (CSFV), a member of the genus *Pestivirus* within the family *Flaviviridae*. It is a highly contagious disease of domestic pigs and wild boars worldwide which results in serious losses in the pig industry. The virulence of CSF viruses is believed to be strain dependent. The mechanisms of CSFV pathogenesis are as yet unknown. It is hypothesized that high and low virulent CSFV strains affect cytokine expression differently. The aim of the current study was to search for elements involved in inducing pathogenic effects of CSFV in the host cell. For this, serial analysis of gene expression (SAGE) and quantitative real-time PCR (RT-qPCR) were used.

SAGE is a sequence-based technique which is designed for global characterization of gene expression patterns in cells. It includes the generation of 14 base long tags originating from the 3' end of each mRNA transcript. It contains sufficient information for the identification of the tag. The construction of concatamers allows cloning and sequencing of the tags. The expression level can be quantified by determination of the number of times a tag is observed [4]. Another possibility to analyze gene expression profiles is the RT-qPCR.

Materials and methods

For the determination of gene expression changes SAGE was used. For SAGE experiments the porcine kidney cell line PK-15 was used. The cells were infected with the highly virulent CSFV strain Kozlov. Five libraries were generated using the MicroSage protocol [3]. PK-15 cells infected with Kozlov were lysed after 2, 6 and 24 hours post infection (pi). Two control libraries from non-infected cells (2 and 24 hours) were included. SAGE tags were submitted to the GenBank porcine EST database (www.ncbi.nlm.nih.gov) using the Blast option to identify the origin.

For RT-qPCR experiments peripheral blood mononuclear cells (PBMCs) were isolated from infected and non-infected pigs and mRNA was isolated and used for RT-qPCR. For the infection of the animals the highly virulent CSFV strain Kozlov, a Bulgarian moderatly virulent strain and the vaccine strain C were used. Blood samples were taken from 0 until 14 days pi. The following cytokines were analysed: interleukin-1β (IL-1β), IL-6, IL-8, tumor necrosis factor-α (TNF-α) and interferon-β (IFN-β). Quantification of cytokine gene expression was performed by the $2^{\Delta\Delta Ct}$ method.

Results

Libraries from infected and non-infected PK-15 cells were compared.

- About 60,000 tags per library were obtained.
- The housekeeping gene β-actin had a similar amount of tags in each of the libraries.
- Transcripts with altered levels could be categorized to code for molecules involved in cellular functions like cellular transport, formation of the cytoskeleton, electron transport chain, protein processing, heat shock proteins and signal transduction.
- For RT-qPCR analysis relative quantities of mRNAs were calculated by $2^{\Delta\Delta Ct}$ method. Normalization of gene expression was carried out by the housekeeping gene β-Actin in infected and non-infected PBMCs.
- First analysis of the samples derived from pigs infected with the Bulgarian moderately virulent strain showed an up-regulation of IL-6, IL-8 and TNF-α after 5 hours pi. After 1 day pi a down-regulation was detected.

Discussion and conclusions

Evaluation of the SAGE gene expression profiles showed that β-actin was expressed at comparable levels in all libraries generated. Therefore this housekeeping gene was used to normalize data in the RT-qPCR studies. Altered gene expression profiles play important roles for some cellular functions including the electron transport chain (e.g. NADH Dehydrogenase), formation of the cytoskeleton (e.g. Tubulin-α), oxidative stress, replication processes, protein processing and signal transduction. Similar results were obtained with infected and non-infected PK-15 cells by proteomic analysis: the identified cytoskeletal proteins like annexin A2 and the antioxidative stress proteins showed an up-regulation after 48 hours pi. Also proteins which are involved in the regulation of the transcription and signal transduction were up-regulated in the proteomic studies. A down-regulation was detected for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and heat shock proteins [2]. In comparison to the 2D gel analysis in the SAGE data cytoskeletal genes like several forms of annexin were detected. One of the most heavily involved annexins in anti-inflammatory responses and apoptotic mechanism is annexin I [1]. Annexin I was up-regulated in infected cells after 24 hours and may play a role in reduction of the inflammation. Transcripts for antioxidative stress genes were also up-regulated at 24 hours pi. These results may indicate that CSFV infection induces host cell oxidative stress. Genes for the regulation of transcription were also identified. Transcripts for histones which play an important role in DNA repair and transcription regulation were up-regulated after 2 hours pi but down-regulated after 24 hours pi. Transcripts for the rho gene which is involved in cell proliferation, apoptosis and multiple other cellular functions were up-regulated in the SAGE library. Analyses of the SAGE libraries from 24 hours pi of CSFV-infected and mock-infected PK-15 cells showed also a down-regulation of GAPDH in the SAGE data like in the 2D gel analysis. The down-regulated transcripts for genes which encode for heat shock proteins may lead to apoptosis because of the incorrect repair mechanism in the host cell.

The analysis of the RT-qPCR data of CSFV-infected PBMCs showed an increase of the gene expression of three of the cytokines analyzed in the first hours after infection, but a decrease after 1 day pi. Far from being complete, further determinations of the level of cytokines expressed in cells from pigs infected with CSFV isolates differing in their pathogenicity may facilitate understanding of the pathogenesis and molecular responses of host cells to CSFV infection.

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THE EFFECT OF INFECTION WITH FELINE CALICIVIRUS ON HOST CYTOSKELETAL PROTEINS

Emma L. NEWSHAM[1,2], Alan D. RADFORD[1], David G. SPILLER[3], Susan DAWSON[1], Rosalind M. GASKELL[1], Jonathan M. WASTLING[2]

[1]*Small Animal Infectious Disease Group, Faculty of Veterinary Science, Leahurst Campus, University of Liverpool, Liverpool, UK*
[2]*Infection Biology, Faculty of Veterinary Science, University of Liverpool, Liverpool, UK*
[3]*Centre for Cell Imaging, School of Biological Science, University of Liverpool, Liverpool, UK*

Keywords: feline calicivirus, proteomics, confocal microscopy

Introduction and Objectives

Throughout virology, genomics techniques have been employed successfully to characterise viruses and visualise their effects on the genes within a cell. There are, however, many fewer examples of proteomic approaches being used to study virus-cell interactions and the effects that virus infections have upon cellular proteins.

Feline calicivirus (FCV) is an important veterinary pathogen with a prevalence between 6-75% within multi-cat colonies (Coyne, Dawson et al. 2006) and it is also a useful model for studying other members of the *Caliciviridae* family which are not readily cultured *in vitro* (Bidawid, Malik et al. 2003*)*.

The initial aim of this investigation was to discover which proteins are differentially expressed by the cell as a result of infection with FCV. The identified proteins could then be subjected to further targeted investigation to discover in which ways the virus is manipulating host cell machinery.

Materials and Methods

Feline embryonic (FEA) cells were infected with FCV-F9 at a multiplicity of infection (m.o.i.) of 10 to create a time course of infection with samples being taken at 1, 4, 7 and 10 hrs post infection (p.i.). These samples were then lysed and the proteins separated and analysed using 2-Dimensional Difference In Gel Electrophoresis (2D-DIGE) which allows differentially expressed protein spots to be identified. These protein spots were then picked out of the gels and tryptically digested before being analysed by Electrospray Tandem Mass Spectrometry (ESI LC-MS/MS) and MASCOT database searching to identify which proteins were picked.

Candidate modulated proteins were then selected for visualisation using confocal microscopy. FEA cells were infected with FCV-F9 at an m.o.i. of 10 and fixed at 1, 4 and 7 hours p.i.. The cells were then stained for vimentin, tubulin and actin proteins and visualised using Alexa-fluor conjugated secondary antibodies 488 and 633 (Sigma-Aldrich). Images were then taken using a Zeiss LSM710 confocal microscope with a Zeiss Fluar 40x 1.3NA objective.

Results

Results from the proteomic analysis showed that a number of cellular cytoskeletal proteins were modulated during the course of infection with FCV-F9. 8 protein spots were upregulated over the course of infection including actin (between 1.5 and 3.5-fold increase), vimentin (3-fold) and tubulin (1.5-fold) and 8 were downregulated including actin (between 1.5 and 4.5-fold decrease), transgelin (9.5-fold decrease) and lamin A (4-fold decrease). From these findings three candidate proteins were chosen for investigation with confocal imaging; vimentin, tubulin and actin. The choice to include these proteins was made on the basis of their cellular abundance for easier visualisation and also the availability of suitable antibodies for use with a feline cell line. Evidence gained from the confocal imaging supports the findings of the proteomics experiment. The pattern of fluorescence of the stained proteins is markedly different between infected and uninfected cells suggesting that major changes within the cytoskeleton do occur during infection.

Discussion and Conclusions

Cytoskeletal proteins are mostly found in three different types of proteins structures which exist independently but interact with each other to perform different cellular functions: microfilaments, microtubules and intermediate fibres (Henderson and Weber 1981). Microfilaments are mainly made up of polymerised actin and are important for maintaining cell morphology. Microtubules consist of α and β-tubulin and perform functions such as organelle movement and transport throughout cells. Intermediate filaments include vimentin and lamins within their structures and are used by the cell to maintain morphology and also to anchor cellular organelles into position.

As viruses are essentially unable to move around the cells unaided (Sodeik 2000), it has be shown that they often use cytoskeletal proteins as a mean of transporting both components and assembled virions around the cell (Smith, Murphy et al. 2003).

In the case of FCV we have found evidence that cytoskeletal arrangement does occur during infection but the extent to which this serves to increase infectivity still remains unclear.

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ROLE OF CD9 FOR THE ENTRY OF *BOVINE VIRAL DIARRHEA VIRUS* INTO THE CELL

Katrin PANNHORST, Stefanie BENDFELDT, Inga GROTHA, Beatrice GRUMMER, Irene GREISER-WILKE

Institute of Virology, Department of Infectious Diseases, University of Veterinary Medicine, 30559 Hannover, Germany

Keywords: bovine viral diarrhea virus, bovine CD9, bovine CD46, tetraspanin, receptor

Introduction and Objectives

Bovine viral diarrhea virus (BVDV) is an enveloped RNA virus which belongs to the genus *Pestivirus* within the family *Flaviviridae*. This family also comprises the genera *Flavivirus* and *Hepacivirus* (*Hepatitis C virus,* HCV). The genus *Pestivirus* includes four established species: BVDV-1 and BVDV-2*, Border disease virus* (BDV), and *Classical swine fever virus* (CSFV)*;* and a tentative species*, Pestivirus of giraffe.* While CSFV seems to be host specific and at least in nature only infects *suidae*, ruminant pestiviruses are known to infect pigs. In addition, some BVDV isolates can multiply in porcine cells [3]. BVDV enters into susceptible cells via clathrin-dependent endocytosis, involving heterodimers of the envelope glycoproteins E1 and E2 [4] and probably also the E^{rns} protein as viral key players. The cellular surface molecule CD46 of bovine cells (boCD46) was identified to be a cellular receptor for BVDV [1]. However, there are strong indications that other molecules are also involved in the multistep process of BVDV entry. Monoclonal antibodies (mabs) directed against the bovine CD9 (boCD9), a member of the tetraspanin superfamily, block BVDV infection of susceptible bovine cells. Tetraspanins are membrane proteins that interact with distinct surface proteins building tetraspanin-enriched microdomains (TEMs) within the cytoplasma membrane. For human CD9 it is known that it associates with CD46 and with other tetraspanins, including the tetraspanin CD81, which was identified as receptor for HCV [2]. The aim of this study is to elucidate the role of boCD9 in infection of cells with BVDV.

Materials and methods

BoCD9 and boCD46 were cloned in pcDNA3.1, and expressed in semi-permissive porcine kidney (SK6) cells. SK6 cells were transiently transfected with pcDNA3.1-boCD9, -boCD46, or with both. Then, cells were infected with the non-cytopathic (ncp) BVDV strain 7443 and the cytopathic (cp) BVDV strain NADL, respectively. After incubation they were fixed and viral antigen was visualized using BVDV specific mabs in indirect peroxidase linked antibody assays (PLA), or by double indirect immunofluorescence (DIF). Quantification of transfected and infected cells was performed by flow cytometry. Virus titrations were performed on fetal bovine kidney (FBK) cells.

Results

To find the optimal conditions for expression of the putative receptor molecules, SK6 cells were transfected with pcDNA3.1-boCD9, -boCD46 or with both and fixed after different incubation times. BoCD9 or boCD46 were detected by indirect PLA or by DIF using CD9 and CD46 specific mabs, respectively. The highest expression rate was observed after 24 h. Then, SK6 and Madin-Darby bovine kidney (MDBK) cells were infected with BVDV ncp 7443 or cp NADL, respectively. Virus was harvested at different times after infection and titrated on FBK cells. As expected, the replication rate of BVDV ncp 7443 or cp NADL in SK6 cells was significantly lower than in MDBK cells, and only discrete plaques were observed.

Then, SK6 cells were transfected with pcDNA3.1-boCD9, -boCD46 or with both. As controls non-transfected cells and cells which were transfected with the empty vector were included. Cells were infected 24 h after transfection with BVDV ncp 7443 at a multiplicity of infection (moi) of 10 or with cp NADL at a moi of 1, respectively. Infection rate was analyzed 24 h, 48 h and 72 h post infection (p.i.) using the PLA. The non-transfected SK6 cells as well as cells which were transfected with the empty vector showed only a small number of plaques. Plaques were also found after transfection with boCD9. After transfection with boCD46 the number of infected cells increased in comparison to the controls, thus confirming earlier results [1]. An additional increase of infected cells was found after co-transfection of boCD9 and boCD46. Currently, these results are being confirmed by flow cytometry.

Discussion and conclusions

Expression of boCD46 increases the infection rate in semi-permissive SK6 cells. An additional increase of the infection rate was found in cells expressing both boCD46 and boCD9; quantification by flow cytometry is in progress.

These results allow to hypothesize two possible functions for CD9. On one hand, the binding of BVDV to the cell and initiation of virus entry occurs via boCD46 in combination with boCD9, which acts as a co-receptor. The fact that semi-permissive porcine cells become permissive after being able to express boCD46 and virus replication is even more efficient when both boCD46 and boCD9 are present indicates that the virus could also use the porcine counterparts, yet with less efficiency. To clarify whether these two proteins are really sufficient to allow virus binding and entry, both proteins will have to be expressed in cells non-permissive for BVDV, i.e. of other species. On the other hand, boCD9 may act after binding of virus particles in penetration or uncoating or even in further steps of BVDV replication, as it was shown for CD9 in the replication cycle of *Feline immundeficiency virus* and *Canine distemper virus* [5]. This will be a challenge for further studies.

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ANALYSIS OF WELL-DIFFERENTIATED PORCINE AIRWAY EPITHELIAL CELLS AS HOST CELLS FOR SWINE INFLUENZA VIRUSES

Darsaniya PUNYADARSANIYA[1], Isabel HENNIG-PAUKA[2], Christine WINTER[1,3], Christel SCHWEGMANN-WESSELS[1], Georg HERRLER[1]

[1]*Institute of Virology, University of Veterinary Medicine, Hannover, Germany;*
[2]*Clinic for swine, small ruminants and forensic medicine, University of Veterinary Medicine, Hannover, Germany*
[3]*Clinic for Poultry, University of Veterinary Medicine, Hannover, Germany*

Keywords: airway epithelium, differentiated cells, lectins, precision-cut lung slices, sialic acid, swine influenza viruses

Introduction and objectives
Influenza viruses initiate infection of their host by binding of the haemagglutinin (HA) to sialic acid residues presented by cell surface components. Viruses isolated from different host species may differ in their preference for the type of sialic acid, e.g. N-acetylneuraminic acid (Neu5Ac), or for a linkage type connecting the sialic acid molecule to the neighbouring sugar. Influenza viruses from mammalian hosts have a preference for α-2,6-linked sialic acids (1). Most avian influenza viruses prefentially recognize Neu5Ac attached via an α-2,3-linkage to galactose. Apart from the linkage type, influenza viruses may also have preferences for different types of sialic acid. Human cell cell contain preferentially N-acetlyneuraminic acid. On porcine cells, N-glycolylneuraminic acid is a prominent type of sialic acid that is completely absent on the surface of human cells

Primary target cells for influenza viruses are epithelial cells lining the respiratory tract. They comprise specialized cells such as ciliated cells or mucus-producing cells that arise after a process of differentiation. Culture systems for such cells have been described and used to analyze infection by human and avian influenza viruses. Comparable studies with porcine airway epithelial cells have not been reported

Materials and methods
Precision-cut lung slices (PCLS) were prepared from the lungs of 3 monthes old pigs. PCLS were stained for the presence of sialic acids using lectins from *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA) To determine the viability of PCLS, slices were analyzed ciliary activity, bronchoconstriction and cell integrity (live/dead staining)

Results
We prepared precision-cut lung slices from the porcine lung by subjecting the *lobus accissorius* filled with low-melting agarose to a tissue slicer. Slices about 250 µm thick contained viable cells for more than one week as determined by monitoring the ciliary activity. A second criterion of vitality was the ability to induce bronchoconstriction by addition of methacholine. This effect was dose-dependent and reversible. As a third criterion of vitality, slices were subjected to live/dead staining. This method indicated that epithelial cells lining the bronchi and bronchioli retained their integrity.

We are interested which of the different types of differentiated epithelial cells are most sensitive to infection by porcine influenza viruses. As sialic acid serves as a receptor determinant for attachment of influenza viruses to the cell surface, we want to determine whether expression of sialic acids in different linkage types reflects the sensitivity of the respective cells to infection by influenza viruses. For this purpose we initiated a lectin staining analysis. The lectin MAA preferentially recognizes α2,3-linked sialic acid, whereas SNA recognizes α2,6-linked sialic acid. Preliminary date indicate that porcine respiratory epithelial cells are more similar to human airway cells than to their counterparts in the avian respiratory tract. While in the chicken respiratory epithelium, almost exclusively, α2,3-linked sialic acid is detected, precision-cut lung slices from the porcine lung contained a substantial amount of α2,6-linked sialic acid. In future experiments, PCLS will be subjected to infection by swine influenza viruses. Infected cells will be stained and compared with the staining pattern obtained with MAA and SNA.

Discussion and conclusions
The recent outbreak of an H1N1 infection in Mexico and its spread to different countries has demonstrated the epidemiological importance of pigs as a host for influenza viruses. Both human and avian strains have crossed the species-barrier to pigs and established different lineages. Reassortment of the gene segments is a constant threat to the generation of viruses with unexpected pathogenicity properties.

As pigs are considered as mixing vessels for avian and human influenza viruses, one might expect that the primary target cells in the respiratory tract contains the receptor determinants for both avian and human influenza viruses, i.e. α-2,3-linked sialic acids and α-2,6-linked sialic acids. Our preliminary date indicate, that this assumption may not be correct. This finding demonstrates the importance of studies with well-differentiated respiratory epithelial cells. Precision-cut lung slices should be helpful to analyze the infection of differentiated epithelial cells. Another culture system for well-differentiated epithelial cells are air-liquid interface cultures. Attempts to establish such cultures for porcine epithelial cells are underway

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INTERFERON INVOLVEMENT IN BORNA DISEASE VIRUS INFECTION

Jonas J. WENSMAN[1], Anne-Lie BLOMSTRÖM[1], Carolina ILBÄCK[1], Elina SANDWALL[1,5], Anders JOHANNISSON[2], Sándor BELÁK[1,3], Anna-Lena BERG[4], Mikael BERG[1]

[1]Swedish University of Agricultural Sciences, Department of Biomedical Sciences and Veterinary Public Health, Uppsala, Sweden;
[2]Swedish University of Agricultural Sciences, Department of Anatomy, Physiology and Biochemistry, Uppsala, Sweden;
[3]National Veterinary Institute, Department of Virology, Immunobiology and Parasitology, Uppsala, Sweden;
[4]Astrazeneca R&D, Safety Assessment, Södertälje, Sweden;
[5]Present address: Uppsala University, Department of Medical Biochemistry and Microbiology, Uppsala, Sweden

Keywords: Borna Disease Virus, interferon, apoptosis, staggering disease

Introduction and Objectives
Borna Disease Virus (BDV) is a neurotropic RNA-virus that persists in cells of the central nervous system (CNS) of a wide range of animal species. To be able to persist, the virus needs to evade the immune response of the host. Major key players of the host immune response are the type I and II interferons (IFN). Type I IFNs induce an anti-viral state in the infected cell and its neighbours, by for example inducing apoptosis, while the type II IFN-gamma plays an important role in the non-cytolytic virus clearance. The mechanisms behind the BDV persistence of the CNS and the immune response in naturally infected animals are poorly understood. In this study, the IFN expression in cats with staggering disease, a neurological disorder linked to BDV infection (3), was investigated. Furthermore, apoptosis resistance in different BDV-infected cell lines was studied.

Materials and methods
Expression of IFN mRNA: Brain tissue of cats with and without clinical and histopathological signs of staggering disease (feline Borna disease (BD)) were used. Total RNA was extracted (Qiagen Lipid Tissue kit) including a DNase treatment step. Fivehundred ng of RNA was converted into cDNA and subsequently used in qPCR. The expression of type I IFNs (IFN-alpha and –beta) and type II IFN (IFN-gamma) was studied using previously published and in-house qPCR-systems (1, 2). The IFN expression was normalised to the expression of three reference genes (4). The presence of BDV RNA was studied using a previously published real-time RT-PCR assay (5).

Resistance to apoptosis in vitro: Rat astrocytoma (C6) and monkey kidney (Vero) cells were infected with BDV strain He/80. To induce apoptosis hydrogen peroxide was added to the cell culture medium and the cells were incubated for 24 or 48 h. The proportion of apoptotic and living cells were measured by flow cytometry.

Results
IFN-gamma was expressed in cats with staggering disease, but no or only low expression was seen in cats without clinical and histopathological signs of staggering disease. The expression of the type I IFNs did not show any significant difference between the two groups of cats.

Further, it was clearly shown that infected C6 cells (C6-BDV) were more resistant to apoptosis than non-infected C6 cells (approximately 11-fold difference). However, the opposite effect was seen in infected Vero cells (Vero-BDV), i.e., Vero-BDV cells were more prone to apoptosis than non-infected Vero cells after hydrogen peroxide treatment (approximately 4-fold difference).

Discussion and conclusions
IFNs play important roles in the immune response of viral infections. BDV causes a persistent infection of the CNS. Hence, BDV needs to be able to evade the host immune response. The studied cats with feline BD have probably been infected for a long period of time since they all were in the end-stage of the disease. The exact duration of infection is, however, not known. Cats are known to have a lower viral load than other species, such as horses. Although not studied, there could be a species difference in immune response. It might be so that cats have a better capability of virus clearance through increased IFN-gamma expression, compared to horses.

The ability to resist apoptosis was tested in an *in vitro* system using different cell lines. Vero cells are known to lack a normal type I IFN system, which unable them to produce type I IFN in response to for example virus infection. C6-BDV is, on the other hand, capable of expressing type I IFN. This difference in type I IFN expression capability could be responsible for the observed difference in apoptosis resistance. However, this data does not reflect the natural situation where there were no significant difference in the levels of expression of type I IFN in the infected cats compared to the controls. A significant increase of type II IFN expression was however seen. It is possible that local production of IFN renders the cells resistant to apoptosis. Another possibility is that type II IFN also is important for resistance to apotosis both *in vivo* and *in vitro*. Thus, our study shows the importance of IFNs in BDV infection, both in natural infection of cats and in apoptosis resistance of cells.

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EVALUATION OF TWO MODIFIED LIVE CSFV MARKER VACCINES IN A COMPARATIVE ANIMAL TRIAL

Andrea AEBISCHER, Martin HOFMANN

Institute of Virology and Immunoprophylaxis, Mittelhaeusern, Switzerland

Keywords: classical swine fever virus, marker vaccine, chimeric pestivirus, DIVA principle

Introduction and Objectives

Classical swine fever (CSF) is a highly contagious disease of swine caused by the classical swine fever virus (CSFV) which belongs to the genus Pestivirus within the family of Flaviviridae.

Despite intensive international efforts in eradicating the disease, CSF is still endemic in some states of the EU and sporadic outbreaks still occur in free areas, causing significant economic losses.

The current control strategy for CSF in the EU is based on stamping out an pre-emptive culling. This approach causes huge economic losses and raises increasing ethical concerns, furthermore, it is not suitable for the disease control in endemic regions and in wild boar populations.

Therefore, systematic prophylactic vaccination with live attenuated marker vaccines has been reconsidered as a promising strategy for the future control and possible eradication of CSF.

In a previous EU research project several new vaccine prototypes were analysed for their potential (3, 5) and two of the most promising candidates have been selected for further evaluation.

The aim of this study was to test those two marker vaccine candidates in a comparative animal trial to collect additional in vitro and in vivo data with regard to safety, potency, efficacy and applicability of the DIVA principle (differentiation between infected and vaccinated animals).

Materials and methods

Both vaccine candidates are prototypes of chimeric pestiviruses. The first one named CP7_E2alf is based on a BVDV I backbone with the antigenic region of the E2 gene replaced by the corresponding sequence of CSFV strain Alfort 187 (2, 3). The second candidate, termed Flc11, is based on the CSFV strain C with the antigenic region of the E^{rns} gene replaced by the respective sequence of BVDV II strain 5250 (1, 4).

Two groups of 6 SPF piglets were orally immunized with the two vaccine candidates and in parallel a control group of 6 animals was vaccinated with standard C-strain Riems. Each group was brought together with 3 non-vaccinated contact animals 2 days post vaccination (d.p.vac.) in order to observe a possible shedding of vaccine virus.

All pigs, as well as 3 additional non-vaccinated control animals, were challenged 14 days d.p.vac. with the highly virulent CSFV strain Koslov.

2 days after challenge one non-vaccinated and non-challenged contact animal was placed among each group to monitor shedding of the challenge virus.

Rectal temperature and clinical signs were recorded on a daily basis and pigs displaying distinct clinical signs of CSF were killed for animal welfare reasons.

The remaining animals were kept until the end of the animal trial 41 d.p.vacc., corresponding to 27 days after challenge infection, when all the pigs were killed and a necropsy was performed.

Blood samples and nasal swabs were obtained two times a week and tissue samples were collected at necropsy.

The samples will be analysed for E2 antibodies, neutralising antibodies, E^{rns} antibodies, and for infectious virus as well as for viral RNA.

Results

Following oral vaccination, none of the vaccinated animals nor any of the non-vaccinated contact animals showed any clinical disease signs. After challenge infection in each group immunised with the two candidate vaccines, 1 out of 6 pigs showed mild clinical symptoms of CSF. Both animals recovered fully after a few days. In contrast, all Riems-vaccinated animals remained completely healthy.

All the non-vaccinated contact and control animals developed severe clinical signs of CSF and had to be killed within 5 to 14 days after challenge. At necropsy those animals displayed a broad range of CSF-specific gross lesions: spleen infarction, enlarged and hemorrhagic lymph nodes, necrosis of tonsils and petechial haemorrhages on kidneys.

Surprisingly, pathological examination of the CP7_E2alf- and Riems-vaccinated, healthy animals 27 days post challenge still revealed infarctions of the spleen and enlarged lymph nodes, whereas none of the Flc11-vaccinated animals displayed any CSF-specific gross lesions.

Diagnostic analyses have yet to be completed, but preliminary results of serological tests show that both candidate vaccines induce a quick (earliest antibody detection after 11 d.p.vac.) and efficient antibody response, comparable to the Riems-vaccinated animals.

Discussion and conclusions

Candidate vaccine Flc11 was for the first time tested upon oral application. It proved to be safe to use, since neither the vaccinated animals, nor the non-vaccinated contact animals, showed any adverse reaction after vaccination. After challenge infection 5 out of the 6 vaccinated animals were fully protected against clinical signs of CSF and at necropsy no CSF-specific gross lesions could be found.

Candidate CP7_E2alf has already been tested in several previous animal experiments and was found to be efficient in domestic pigs and in wild boar after intramuscular and oral application. In our study the vaccine proved to be safe to use, but in contrast to the previous experiments, it did not protect all the vaccinated animals against clinical signs of CSF after challenge with the highly virulent strain Koslov. Furthermore, all the vaccinated pigs revealed distinct pathological lesions at necropsy.

However, these results will have to be further evaluated in combination with data from the virological and serological analyses.

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TARGETING TO ANTIGEN-PRESENTING-CELLS BY DNA VACCINATION INDUCES PROTECTIVE RESPONSES AGAINST FOOT-AND-MOUTH DISEASE IN SWINE

Belén BORREGO[1], Jordi M. ARGILAGUET[2], Eva PÉREZ-MARTÍN[2], Mariano PÉREZ-FILGUEIRA[3], José M. ESCRIBANO[3], Francisco SOBRINO[1,4] & Fernando RODRÍGUEZ[2]

[1] CISA-INIA, Valdeolmos, Madrid, Spain;
[2] CReSA, UAB, Bellaterra, Barcelona, Spain;
[3] Dpto de Biotecnología INIA Madrid, Spain;
[4] CBMSO, UAM Cantoblanco, Madrid, Spain

Keywords: Foot-and-Mouth Disease Virus, DNA vaccines, Antigen Targeting, Cellular Response

Introduction and objectives

Foot-and-Mouth Disease (FMD) is one of the most devastating diseases for animal health, and development of novel, safer marker vaccines is essential to avoid the problems associated to the inactivated virus vaccines currently in use. DNA vaccination has become a promising alternative because of its several advantages over conventional vaccines, especially because of the feasibility of manipulating the DNA constructs in order to apply strategies for optimisation and/ or modulation of the immune responses induced. One of the strategies already shown to enhance the immunogenicity of antigens is its fusion to targeting molecules in order to direct the antigen to antigen-presenting-cells (5). Following this approach, we have fused a previously assayed combination of B- and T-cell epitopes from FMD virus (1,3) to a new targeting molecule: APCH I, a single chain variable fragment (scFv) of a monoclonal antibody that recognizes the Class II Swine Leukocyte Antigen (2). Previous experiments with mice immunized with a pCMV plasmid coding for this construct showed that it was able to induce a strong cellular response (1). The vaccine potential of the construct for natural hosts for FMDV was evaluated in a DNA-immunization + viral challenge experiment in swine, aiming also to get an insight into immune mechanisms playing a role in protection against FMDV. In this report we show the results of this experiment.

Materials & methods

4 eight-week old pigs were immunized with the construct pCMV-APCH I-BTT. One pig inoculated with an irrelevant pCMV plasmid was included as control. After receiving three doses of DNA (400 µg), animals were needle-challenged with FMDV-CS8c1. Progression of disease was evaluated daily using a clinical score based on a semi-quantitative rating of clinical signs. Samples collected at 0, 2, 3, 6, 8 and 10 days post challenge were analyzed to determine: FMDV-specific antibodies in sera by ELISA and neutralization tests and virus titer in blood and swabs (both by virus isolation and RT-PCR). Peripheral blood mononuclear cells (PBMCs) were in vitro stimulated with specific antigens and lymphoproliferation assays, IFN-gamma ELIspot and IFN-gamma and IL10 ELISAs were performed.

Results and discussion

PIGS IMMUNIZED WITH PCMV-APCH I-BTT ARE PROTECTED AGAINST FMDV CHALLENGE. After viral challenge, two of the four DNA-vaccinated pigs showed no signs of disease during the 10 monitored days. In agreement with the lack of signs of disease, no virus could be detected at any time and sample from these two pigs clinically protected. Progression of disease for the other two animals was delayed, showing a shorter period of acute disease and milder clinical signs when compared to the control animal. In spite of this, viraemia titres and kinetics in these pigs were similar to those recorded in control animal. Remarkably, viral detection in swabs from these animals resulted mainly negative, suggesting a limited replication of virus unable to reach these epithelia. Reduction in viral replication was also supported by the low titres of antibodies to the non-structural FMDV antigens, with no seroconversion at all to NSP in clinically protected pigs.

PROTECTED ANIMALS RAPIDLY DEVELOP NEUTRALIZING ANTIBODIES. At the time of challenge, neither total anti-FMDV antibodies by ELISA nor neutralizing antibodies were detected in any serum sample from the DNA vaccinated pigs, thus indicating that the protection afforded was mediated by cell-mediated immunity. Surprisingly, in 3 of the 4 pigs immunized with the APCH I-construct, first detection of neutralization occurred at day 3, with low although significant titers (PRN50 between 1.0-2.0). Even though day 3 after exposure to virus seemed to be too early for an specific antibody response, neutralization curves obtained for FMDV isolates belonging to different serotypes and subtypes suggested that this early neutralization was antibody mediated.

PBMCS FROM PIGS IMMUNIZED WITH PCMV-APCH I-BTT ARE ABLE TO PRODUCE IFN-GAMMA AFTER SPECIFIC STIMULATION. Prior to challenge, animals inoculated with the APCH I-construct showed a significant number of cells that specifically secreted IFN-gamma in response to stimulation with inactivated virus. IFN-gamma is known to display a potent antiviral activity on FMDV (4), therefore an effective priming of cells capable to produce this cytokine in response to specific stimulation as observed in our DNA-vaccinated animals may be an important early line of defence that, in the absence of neutralizing antibodies, may prevent viral replication and protect from disease.

Discussion and conclusions

Despite much work remains to be done in order to elucidate immune mechanisms mediating the protection against FMDV infection achieved in pigs immunized with our DNA construct pCMV-APCH I-BTT, the results presented here show that, in the absence of neutralizing antibodies induced by vaccination, priming of specific cells able to respond to the encounter of virus by secreting IFN-gamma, combined with a rapid production of specific neutralizing antibodies after viral challenge, may be crucial in blocking viral replication and providing protection. On the other hand, since cellular responses are expected to be of broader spectrum than those based on antibodies, these results open the possibility of inducing heterotypic protective responses, which would be a main goal in vaccination against FMDV, due to the high variability of FMDV strains.

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COMPARED CROSS-IMMUNOGENICITY OF DIFFERENT AVIAN INFLUENZA H5 HEMAGGLUTININS USING A DNA VACCINATION MODEL IN MUSCOVY DUCKS

Olivier GUIONIE[1], Marie-Odile LE BRAS[1], Stéphanie BOUGEARD[2], Michel BUBLOT[3] and Véronique JESTIN[1]

[1] *AFSSA, Avian and Rabbit Virology Immunology and Parasitology Unit (VIPAC), BP53, 22440 Ploufragan, France;*
[2] *AFSSA), Epidemiology and Quality Assurance in Pig Production Research Unit (EBEP), 22440 Ploufragan, France;*
[3] *Merial, S.A.S, Department of Virology, Discovery Research Direction, merail Lyon Gerland Laboratory, 254 rue Marcel Mérieux, 69007 Lyon, France*

Keywords: avian influenza, hemagglutinin H5, H5N1highly pathogenic, DNA immunization, immunogenicity, Muscovy ducks.

Introduction and Objectives

H5N1 highly pathogenic avian influenza [HPAI] remains enzootic in a few countries in Asia and Africa. It is therefore a major threat for animal health and public health given the zoonotic potential of the viruses. At the same time the circulation of H5 low pathogenic virus [LPAIV] is an important issue in a few countries notably in Europe, given their potential to mutate into HPAIV. In both occasions ducks can play a major role in the epidemiology of AI since wild waterfowl, including ducks, constitute the natural reservoir of all subtypes of influenza A. To control H5 AIV spread, vaccination can be useful provided vaccines are able to induce satisfactory immunity in ducks, notably encompassing the variability of the hemagglutinin (HA), the major immunogen. In fact, several subclades of H5N1 viruses have been reported and H5 LPAIV are phylogenetically distinct from the previous ones. So assessing the cross-immunity induced by selected HA proteins is very important. HA genes belonging to LP and HPAIV were used to vaccinate Muscovy ducks. Cross-immunogenicity of HA specific serum antibodies was studied using hemagglutination inhibition [HI] and neutralisation [SN] tests using several AI antigens.

Material and methods

Different genes from H5N1 and H5N2 LPAIV and from H5N1 HPAI (subclade 2.2) french isolates were cloned into pcDNA 3.1 vector. Synthetic genes with codons optimized for chicken usage were also used: a HA gene from HPAIV A/chicken/Indonesia/7/2003 (clade 2.1), a HA gene from HPAIV A/turkey/Turkey/1/05 (clade 2.2.1), a HA gene from HPAIV A/chicken/Laos/3295/06 (clade 2.3.4). All cleavage sites of the HA genes were modified to match that of LP strains. Protein expressions were checked by transient expression and indirect immunofluoresence after cell transfection. DNA immunization was realized on 5-weeks-old specified pathogen free Muscovy ducks using a needle free device. A boost was done 3 weeks after the prime and the bleeding 3 weeks after boost. Immunogenicity of the corresponding expressed proteins was compared by HI and SN using LP and HP antigens belonging to clade 2.2, the only clade detected in Europe until now. All differences between means were analysed using Student-Newman-Keuls test.

Results

100 µg was the optimal dose for DNA vaccination. HA gene from LPAIV gave high HI and SN titres using LP antigens (SN titres being higher than HI titres using homologous antigen), but very low titres using HP clade 2.2 antigens. All HP genes gave high titres using 2 strains from HPAIV clade 2.2 (including one classified as 2.2.1). The optimized HA gene from HPAIV A/turkey/Turkey/1/05 gave the highest HI and SN titres, when using antigens belonging to the same clade (2.2). On the other hand, HA gene from A/duck/Laos/3295/06 (clade 2.3) presented the lowest immunogenicity against clade 2.2.

All unoptimized HA genes gave low HI and SN titres using LP antigens, whereas all optimized HA genes gave good titres using LP antigens, the highest titres being obtained using HA gene from A/duck/Laos/3295/06 (clade 2.3).

In order to determine if the good cross reactivity of optimized HP HA genes with LPAI antigens was due to the synthetic nature of their nucleotide sequences, data obtained with unoptimized and optimized HA genes belonging to the same clade (2.2) were compared. It resulted that titres using optimized HA gene were statistically higher than those obtained with the unoptimized gene, showing the effect of the synthetic nature of HA gene on immunogenicity.

Discussion and conclusions

All these data suggest that subclade 2.2.1 optimized HA gene from A/turkey/Turkey/1/05 appeared to be the best candidate to be inserted in a recombinant vaccine since it induced cross immunogenicity against AIV circulating in Europe nowadays. However it remains necessary to check whether this cross-immunogenicity also affords a cross-protection on ducks.

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PUTATIVE ANTIGENIC DOMAINS ON THE E^{RNS} PROTEIN OF *PESTIVIRUSES*

Denise HENRYCH, Volker MOENNIG, Irene GREISER-WILKE

Institute of Virology, Department of Infectious Diseases, University of Veterinary Medicine, 30559 Hannover, Germany

Keywords: *Classical swine fever virus*, structural glycoprotein E^{rns}, epitope mapping

Introduction and Objectives

Classical swine fever (CSF) is a highly contagious disease of domestic pigs and wild boar and is caused by the *Classical swine fever virus* (CSFV). CSFV is a small enveloped virus containing a single stranded RNA genome with a length of approximately ~12.3 kb. Together with the *Bovine viral diarrhea virus* (BVDV) and the *Border disease virus* (BDV) it forms the genus *Pestivirus* within the family *Flaviviridae.* The E2 and to a lesser extent the E^{rns} proteins induce neutralizing antibodies which confer protective immunity, thus being candidates for the development of vaccines. ELISAs detecting the antibodies directed against the E^{rns} protein have been found to allow discrimination between vaccinated and infected animals (DIVA) when the corresponding subunit vaccine consisting of the CSFV E2 glycoprotein is used. For the development of a new marker vaccine and the corresponding DIVA tests it is important to determine which epitopes are involved. To supplement the existing knowledge concerning the antigenic domains and epitopes of the CSFV- and BVDV E^{rns} protein, chimeric and truncated constructs were designed and the reactivity of 13 monoclonal antibodies (mabs) was analyzed. Furthermore the cross reactivity of the mabs was determined to obtain information concerning the conservation of epitopes on the E^{rns} proteins of *Pestiviruses*.

Materials and methods

The full length gene of the E^{rns} from CSFV strain Alfort/187 and BVDV strain NADL, respectively, were cloned into the pCITE plasmid, and four chimeric E^{rns} constructs with combinations of fragments of each of the genes were generated. In addition a truncated E^{rns} construct coding for the amino acids (aa) 55 to 110 was constructed. After transfection into BSR T7 cells (modified baby hamster kidney cells, stably expressing the T7 polymerase) reactivity with the chimeric proteins was determined in peroxidase linked antibody assay (PLA) using a panel of 13 mabs (eight mabs were raised against the CSFV strain Alfort/187 and five mabs against the BVDV strain NADL). To confirm the suitability of the chimeric constructs for epitope mapping sequence alignments (software Align Plus 4) and protein modeling studies (software Modeller; [2]) of the corresponding proteins of BVDV strain NADL and CSFV strain Alfort/187 were performed. The homology modeling was based on the E^{rns} model of the CSFV strain Brescia [1]. To determine the cross reactivity of the mabs susceptible cells were infected with a panel of different pestiviruses. The mabs were titrated in a PLA and the activity factors (AF = titer in PLA/mg protein per ml) were calculated.

Results

The amino acid sequence identity between the E^{rns} protein of CSFV strain Alfort/187 and BVDV strain NADL is 73%. Structural elements like cysteins involved in intramolecular disulfid bridges and potential glycosylation sites are conserved in both proteins and in the chimeras. All mabs tested reacted with at least one of the chimeras. Some mabs were directed to one antigenic domain on the CSFV E^{rns} protein and to one on the BVDV E^{rns} protein, respectively. Four mabs bound to all chimeras. An antigenic domain could not be assigned to these mabs. None of the mabs showed a reaction with the truncated E^{rns} protein (aa 55-110). Furthermore most mabs were CSFV or BVDV specific, respectively.

Discussion and conclusions

Computer-based structural analysis indicated that important motifs were conserved on the E^{rns} proteins of CSFV strain Alfort/187 and the BVDV strain NADL. This was substantiated by the fact that four mabs bound to all chimeric proteins and the other mabs reacted with their epitopes each on one of the chimeras. Therefore the folding of the chimeric E^{rns} proteins seems to be similar to the native protein. The mabs allowed the identification of one antigenic domain on the CSFV E^{rns} protein (aa 55-110) and one on the BVDV E^{rns} protein (aa 111-167). The linear epitope DKN (aa 117-119) [3] was not detected by any of the mabs.

None of the mabs bound to the truncated E^{rns} protein (aa 55-110). This result could be due to the fact that the folding of the truncated protein might be different in comparison to the folding of the native E^{rns} protein. Another reason for this could be that conserved amino acids beyond the antigenic domain (aa 55-110) might also be important for antibody binding. This indicates that the epitopes must be conformational. Most mabs were CSFV or BVDV specific which indicates that the epitopes seem to be conserved within the genera. Construction of new chimeric or truncated proteins and site-directed mutagenesis will be applied to further chracterize the epitopes.

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ANTIGENIC VARIATION IN ERBOVIRUSES

Jacquelyn HORSINGTON, James GILKERSON, Carol HARTLEY

Equine Infectious Diseases Laboratory, Faculty of Veterinary Science, University of Melbourne, Australia,

Keywords: erbovirus, antigenic sites

Introduction and Objectives

Respiratory infections are a major concern in horses worldwide, particularly within the multi-million dollar horse racing industry. One virus associated with equine respiratory disease is equine rhinitis B virus (ERBV), the sole species in the *Erbovirus* genus of the family *Picornaviridae*. ERBV has been regularly isolated from horses displaying clinical respiratory disease and ERBV neutralising antibodies have been detected in 50-80% of horses around the world (Steck *et al.,* 1978; Carman *et al.,* 1997). ERBV has three distinct serotypes with both acid-labile and acid-stable phenotypes (Huang *et al.,* 2001; Black *et al.,* 2005). To date little is known of the antigenic structure and pathogenesis of the virus. The objective of this study was to investigate the antigenic structure of ERBV capsid proteins and identify the contribution of virus proteins to serotype specificity.

Materials and methods

Viral genome sequences coding the capsid proteins were examined and distinct regions of variation between serotype within the capsid proteins VP1 and VP2 were identified. Five target proteins from each serotype linked to either glutathione-*s*-transferase (GST) or a hexa-histidine tag were expressed in *E.coli* and purified in native form. Proteins were tested for reactivity to serotype specific rat sera.

Results

The full length VP1 and VP2 proteins of ERBV1, ERBV2 and ERBV3 were expressed with a hexahistidine tag and probed with serotype specific rat sera in Western blots. VP2 from each virus serotype cross-reacted with each serum however, there was no cross-reaction between serotypes with VP1 which only reacted with sera from rats immunised with a homologous serotype. To define serotype specific regions within VP1 and VP2, additional fusion proteins were constructed. Regions of VP1 and VP2 that showed the highest level of variability between serotypes were expressed as GST fusion proteins. Western blots probed with serotype specific rat sera and found VP1-GH and VP1-Ct reacted only with sera from rats immunised with a homologous serotype, with no cross-reaction between serotypes. Similarly, VP2-EF reacted only with sera from rats immunised with a homologous serotype, with no cross-reaction between serotypes.

Discussion and conclusions

VP2 contains B cell epitopes that cross-react across the three erbovirus serotypes, and the cross-reactive epitopes are contained in regions other than the VP2 EF loop. VP1 contains serotype specific B cell epitopes, at least some of which locate to the GH loop and C-terminal region of VP1. This is consistent with the notion that VP1 contains virus neutralising epitopes and significantly contributes to defining the serotypes of these viruses. Reactivity of these fusion proteins to serotype specific sera from naturally infected horses will also be examined. The suitability of these proteins as antigens in an ELISA to test horse sera will be investigated.

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IMPROVE TOOLS AND STRATEGIES FOR THE PREVENTION AND CONTROL OF CLASSICAL SWINE FEVER (CSFV_GODIVA)

Frank KOENEN[1], Andy HAEGEMAN [1], Åse UTTENTHAL[2], Marie-Frédérique LE POTIER[3], Gábor KULCSÁR[4], Willie LOEFFEN[5], Juan Plana DURÁN[6], Martin BEER[7], Gian Mario DE MIA[8], Sophie ROSSI[9], Rong GAO[10], Sándor BELÁK[11], Volker MOENNIG[12], Jose SANCHEZ-VIZCAINO[13], Hans-Hermann THULKE[14], Niels DELATER[15], Paolo CALISTRI[16], Martin HOFMANN[17]

[1]Veterinary and Agrochemical Research centre, Brussels, Belgium;
[2]National Veterinary Institute, Technical University of Denmark, Lindholm Denmark;
[3]Agence Française de Sécurité Sanitaire des Aliments, Ploufragan, France;
[4]Central Agricultural Office, Directorate of Veterinary Medicinal Products, Budapest , Hungary;
[5]Central Veterinary Institute of Wageningen UR, Lelystad, The Netherlands;
[6]Fort Dodge Veterinaria SA, Vall de Bianya (Girona), Spain;
[7]Friedrich-Loeffler-Institut, Island of Riems, Germany;
[8]Istituto Zooprofilattico Sperimentale Umbria e Marche, Perugia, Italy;
[9]Office National de la Chasse et de la Faune Sauvage, Paris, France;
[10]Bioengineering Research Centre for Animal Disease Prevention and Control, Key Laboratory for Bio-Resource and Eco-Environment of Ministry of Education, Sichuan University, Chengdu, China;
[11]The National Veterinary Institute, Uppsala, Sweden;
[12]Institute of Virology, School of Veterinary Medicine Hannover, Hannover, Germany;
[13]Universidad Complutense de Madrid (Sanidad Animal), Madrid, Spain;
[14]Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany;
[15]Spectos GmbH, Dresden, Germany;
[16]Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G.Caporale", Teramo, Italy;
[17]Institute for Virology and Immunoprophylaxis, Mittelhäusern, Switzerland

Keywords: back yard pigs, classical swine fever, diagnosis, epidemiology, live marker vaccine, wild boar

This presentation summarizes the aims and objectives of a newly started CSFV project.

Although classical swine fever (CSF) has been eradicated in wide areas within the EU, the disease is still endemic in some wild boar populations and in domestic pigs in some of the new member states, particularly in back yard pigs. This increases the risk of CSF re-emergence and persistence in pig farms.

In order to improve the eradication strategies the project aims are a) the final development and testing of a live marker vaccine candidate for the prevention and improved control of CSF, applicable both orally and intramuscularly; b) the development and optimisation of accompanying discriminatory diagnostic tests; c) the production of an effective oral delivery system for the marker vaccine for use in wild boar and back yard pigs; d) the easy selection of diseased animals using infra red temperature measurement; e) the evaluation of strategy concepts to control outbreaks in the wild or domestics while accounting for the new tools.

The improved knowledge on immunological reactions and pathogenesis will support a more efficient vaccine application and provide data for the epidemiological models.

In addition, research about bait-uptake by wild boar will allow increasing the efficacy of oral vaccination.

Epidemiological studies of CSF in domestic pigs, back yard pigs, and in wild boar, including molecular epidemiology will increase the insight of CSF transmission and persistence. Epidemiological models will be developed to support risk assessment, both for conventional eradication strategies as well as for new strategies using the new vaccines and diagnostic tools taking into account the role of CSF reservoirs.

A proof of concept concerning the suppression of viral replication by alternative methods will also be provided.

In conclusion, the new EU-funded project will provide the scientific basis for new strategies of eradication and control of CSFV in the European Union.

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IN VITRO AND *IN VIVO* CHARACTERISATON OF BOVINE VIRAL DIARRHEA VIRUS (BVDV) N^{PRO} DELETION MUTANTS

Patricia KÖNIG, Johanna ZEMKE, Katrin MISCHKALE, Ilona REIMANN, Martin BEER

Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Federal Institute for Animal Health, Greifswald-Insel Riems, Germany

Keywords: BVDV, vaccine safety, Npro

Introduction and objectives

BVDV causes major economic losses due to reduced fertility, abortions, and the generation of persistently infected calves, which may develop fatal "Mucosal Disease". The two species of Bovine viral diarrhea virus, BVDV-1 and BVDV-2 are grouped in the genus Pestivirus within the family of *Flaviviridae* (Fauquet *et al*., 2005). Vaccination is an important tool for BVDV-control, claiming not only clinical protection but also prevention from fetal infection in pregnant animals. Protection against BVDV-2 infection after vaccination with commercially available BVDV-1 vaccines is often limited due to the marked genetic and antigenic heterogeneity between BVDV-1 and BVDV-2 (Becher *et al*., 1999; Beer *et al*., 2000). However, for the application of highly effective modified live vaccine viruses, the safety of commercial live vaccines relating to fetal infection is under discussion. The viral N-terminal autoproteinase N^{pro} counteracts interferon (IFN)-α/-β induction and mediates evasion of the interferon response in infected cells. Therefore, BVDV N^{pro} deletion mutants are attenuated and may presumably induce a self-limiting infection in the developing fetus already in early stages of the gestation by stimulation of the innate immune system (Charleston *et al*., 2001).

In this study, novel BVDV type 1 and type 2 N^{pro} deletion mutants were characterised in cell culture and in vaccination-challenge experiments in calves.

Material and methods

Based on infectious full-length cDNA clones, BVDV-1 and -2 N^{pro} deletion mutants were constructed. Cell culture properties of the recombinants were determined on different cell lines. Interferon secretion and induction of apoptosis, as well as growth characteristics and plaque sizes were evaluated. Bovine calves were intramuscularly vaccinated with a single dose of approximately 6 $\log_{10}$ $TCID_{50}$ of the type 1 or type 2 N^{pro} deletion mutants or were simultaneously or sequentially immunised with a combination of ΔN^{pro} mutants of both BVDV subtypes. BVDV-1 or -2 challenge virus was intranasally instilled with a nebulizer. After immunisation as well as after challenge infection the animals were monitored for body temperatures, clinical disease, leukocyte counts, viremia and nasal virus excretion over a period of 10 to 14 days.

Results

All N^{pro} deletion mutants could be propagated on conventional cells at high titers. However, the recombinants showed a clear growth delay in cell culture with a moderate to low reduction of the final titers compared to the parental viruses. This growth deficiency as well as an accompanying reduction of the plaque sizes could not completely be restored on interferon incompetent cells. These findings indicate an additional interferon independent growth impairment which might be due to the aminoterminal fusion of a 12 aa comprising residue of the N^{pro} protein to the capsid proteins of the N^{pro} deletion mutants.

As determined by Vesicular stomatitis virus (VSV) plaque reduction assays, all N^{pro} deleted viruses induced secretion of biologically active interferon into the cell culture supernatants. A graduated interferon induction was observed with the highest levels in primary-near cells, intermediate levels in diploid cells, and significantly lower amounts in immortalised cell lines.

After immunisation of calves, no adverse effects or alteration of the general health status were observed.

Following challenge infection in mock-vaccinated control animals a severe and prolonged leukopenia was evident while viremia and nasal shedding of challenge virus were observed for 7-12 days. The control animals exhibited fever, nasal discharge, reduced feed intake, and depression.

Depending on the respective N^{pro} deletion mutant, protection at varying levels in regard of clinical disease, viremia and nasal virus excretion was achieved. Even the least efficient vaccine candidate provided remarkable clinical protection and significant reduced amount and duration of challenge virus shedding and viremia. Our non cytopathogenic (ncp) as well as our cytopathogenic (cp) BVDV-1 N^{pro} deletion mutant conveyed sterile immunity against a virulent BVDV type 1 challenge infection. Application of ncpBVDV-2ΔN^{pro} or cpBVDV-1ΔN^{pro} combined with ncpBVDV-2 ΔN^{pro} mediated effective immunity against a highly virulent BVDV type 2 test infection. Furthermore, type 1 challenge experiments after immunisation with the latter viruses are scheduled.

Discussion and conclusions

The novel BVDV N^{pro} deletion mutants were shown to be highly attenuated and efficacious modified live vaccine candidates with the potential to induce sterile immunity against virulent BVDV type 1 and type 2. Enhanced vaccine safety without the risk of persistent infection of the fetus has been demonstrated after infection of pregnant heifers with the ncp and with the cp BVDV-1 N^{pro} deleted virus and will be verified by intra-amniotic application of the respective recombinants as well as of the BVDV-2 ΔN^{pro} vaccine candidates.

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PROTECTIVE EFFICACY OF FMDV SUBUNIT VACCINE PRODUCED USING A SILKWORM-BACULOVIRUS EXPRESSION SYSTEM AGAINST TWO CHINESE TYPE ASIA I ISOLATES IN CATTLE

Zhiyong LI[1], Xiangping YIN[1], Yongzhu YI[2], Baoyu LI[1], Xuerui LI[1], Xi LAN[1], Zhifang ZHANG[2], Jixing LIU[1]

[1]State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Animal Virology of Ministry of Agriculture, Lanzhou Veterinary Research Institute, Chinese Academy of Agriculture Sciences, 1 Xujiaping, Lanzhou, China
[2] Biotechnology Research Institute, ChineseAcademy of Agricultural Sciences, 12 Zhongguancun South Street, Beijing, China

Keywords: foot-and-mouth disease virus; silkworm-baculovirus expression system; subunit vaccine, PD_{50} test

Introduction and objectives

In March 2005, the first incidence of FMDV type Asia 1 was reported in China and two Chinese outbreak strains, Asia1/HNK/CHA/05 and Asia1/JS/CHA/05, were isolated. We have previously demonstrated that a single vaccination with an empty capsids vaccine from FMDV serotype Asia I/HNK/CHA/05, expressed by a silkworm-baculovirus expression vector, can induce protection in 80% of cattle (4/5 animals) when challenged 28 days later with a homologous virus. Here, we designed an experiment to verify whether this produced antigen for protection against the two most prevalent strains, Asia1/HNK/CHA/05 and Asia1/JS/CHA/05.

Materials and methods

The 50% bovine protective dose (PD_{50}) test was performed to assess the potency of the subunit vaccine against two different epidemic isolates of FMDV type Asia I virus in China. Three groups of 10 cattle per group were immunized by intramuscular inoculation (I.M.) in the neck with different doses (1 dose of vaccine, 1/3rd dose of vaccine, or 1/9th dose of vaccine). Three subgroups of five cattle per subgroup and a control subgroup of two animals in group 1 were challenged 21 dpv with 10,000 BID_{50} of FMDV Asia I/HNK/CHA/05. The other animals in group 2 were challenged with 10,000 BID_{50} of FMDV Asia I/JS/CHA/06.

Results

When challenged with two Chinese isolates, the subunit vaccine could achieve 6.47PD_{50} (challenged with Asia I/HNK/CHA/05 strain) and 5.20 PD_{50} (challenged with Asia I/JSL/05 strain) per dose.

Conclusion

The results suggest that it is feasible to use this empty capsid vaccine for preventing Asia I-type FMDV infection.

Acknowledgements

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IMPORTANCE OF INDIVIDUAL NEUTRALISING EPITOPES OF FOOT-AND-MOUTH DISEASE VIRUS IN RELATION TO POLYCLONAL RESPONSE OF VACCINATED ANIMALS

Mana MAHAPATRA, Pip HAMBLIN, David PATON

Institute for Animal Health, Pirbright Laboratory, Ash Road, Woking, Surrey, GU24 0NF, United Kingdom

Keywords: foot and mouth disease virus, neutralising epitopes, VNT, cELISA

Introduction and Objectives

Foot-and-mouth disease (FMD) is a severe, clinically acute vesicular disease of cloven-hoofed domestic and wild animals with a global distribution. The humoral immune response has been generally accepted as the most important factor in conferring protection against the disease, as a strong correlation has been reported between virus neutralising antibody and protection in cattle. Five neutralising antigenic sites (site 1-5) have been identified in serotype O FMD viruses by sequencing monoclonal antibody (mAb) neutralisation escape mutants. Antigenic site 1 has been historically considered to be immunodominant due to evidence implicating its importance in the induction of a protective immune response. In the case of serotype O viruses, using a mAb-based c-ELISA, Samuel (1997) reported the presence of relatively higher titres of site 2 specific antibody in some cattle. In contrast, using mAbs to antigenic site 1-3 in cELISA, Aggarwal and Barnett (2002) reported that none of these antigenic sites (site 1-3) can be considered as immunodominant in a polyclonal serum. In this study we have addressed this question using site-specific mAb neutralisation escape mutants to quantify the relative amounts of antibodies against the known neutralising antigenic sites of FMDV in bovine polyclonal serum.

Materials and methods

MAb escape mutants derived from the O_1 Kaufbeuren strain of FMDV with mutations affecting each of five different antigenic sites were used in this study. Polyclonal antisera were obtained from cattle vaccinated 21 or 28 days previously with inactivated FMD vaccines – either O_1 Kaufbeuren or the closely related O_1 Lausanne and O_1 BFS strains. The titres of neutralising antibodies present in the polyclonal antisera were measured against the original O_1 Kaufbeuren virus and various mAb escape mutant virus descendents by micro-neutralisation test. Titre differences between an antiserum's reactivity with the parental and mutants viruses was taken as an indication of the proportion of antibody reacting to the mutated site.

Results

Polyclonal sera collected from FMD vaccinated animals with an in vitro virus neutralising antibody titre of approximately log 2 (n=22) against O1 Kaufbeuren virus were used for further study. Measurement of neutralising antibody titre differences between parental and various mAb escape mutant viruses revealed a varied immune response in animals; antibodies were detected against all the antigenic sites to various degrees (more antibodies against site 1 and 2 and comparatively less antibodies against site 3-5). 82% of the animals exhibited relatively higher amount of neutralising antibodies against site 2 whereas 18% had a higher amount of antibodies against site 1.

Discussion and conclusion

This study revealed that site 2 appears to be immunodominant in serotype O FMDV in cattle. This is in agreement with the report of Samuel (1997) and also with the results of production of mAbs, where following immunisation of mice with killed whole FMD virus preparations mAbs against antigenic site 2 frequently predominate (unpublished observation). In contrast this does not corroborate with the report of Aggarwal and Barnett (2000) who used mAbs to sites 1-3, some homologous and some heterologous. Though a competition ELISA based approach has been successfully used in various studies involving FMDV, competition has been reported to be dependent on the affinity of the mAbs and, sometimes competition might be observed due to stearic interference because of the large size of the antibody molecule and the relative proximity between the antigenic sites on the surface of FMDV thereby showing different result which may not be true and reliable.

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ALTERNATIVE TESTING OF VIRAL VACCINES FOR EXTRANEOUS AGENTS: DETECTION OF EGG DROP SYNDROME VIRUS BY PCR

Enikő PETROVSZKI, Tibor FARKAS, Erzsébet SOMBOR, Zoltán PÉNZES

CEVA-PHYLAXIA Veterinary Biologicals Co. Ltd., Budapest, Hungary

Keywords: extraneous agent test, PCR, egg drop syndrome virus

Introduction and Objectives

The production of veterinary vaccines in Hungary (including the CEVA-Phylaxia Veterinary Biologicals Co. Ltd.) is regulated by the European Pharmacopoeia (Ph. Eur.) currently in force (1). This document is a collection of standardised specifications on the quality of pharmaceutical preparations and it provides a proper standard for controlling production and ensuring the purity, safety, potency, and efficacy of vaccines. The corresponding monographs of the Ph. Eur. are updated regularly by the authorities of the Council of Europe (European Directorate for the Quality of Medicines & HealthCare) in order to incorporate the latest advances of the field. One important requirement for the veterinary vaccines is to provide the necessary guarantees of freedom from contamination with extraneous agents (EA). Until recently, the testing of avian live virus vaccines (seed lots, batches of finished products) for EAs has been based on detection methods using chicks, embryonated chicken eggs and cell cultures. However, Ph. Eur. allows the application of alternative methods, such as polymerase chain reaction (PCR), for EA testing if they comply with the quality requirements: "other types of tests than those indicated may be used provided they are at least as sensitive as those indicated and of appropriate specificity. Nucleic acid amplification techniques (NAT) give specific detection for many agents and can be used after validation for sensitivity and specificity".

Egg drop syndrome (EDS) is a viral disease that was first described in chickens in the 1970s, and it is characterized by reduction in egg production and egg(shell) quality. The disease is mainly of economic importance as the birds infected with EDS have no clinical symptoms. Although the disease practically affects only broiler breeder chicken flocks, the causative agent of EDS was found to infect other bird species, too, including ducks and geese that appear to be the natural reservoir species of the virus. The etiologic agent of the disease, duck adenovirus 1 (DAdV 1) or commonly known as EDS virus (EDSV), is classified into the genus *Atadenovirus* in the family *Adenoviridae*. Despite its eradication from the majority of commercial breeder flocks, EDSV (DAdV 1) became endemic in chickens in many parts of the world.

The aim of the study was to develop/adopt a PCR assay for the specific detection of EDS virus (DAdV 1) from batches of finished products and to replace the currently used conventional extraneous agent test method.

Materials and methods

Prior to the comparative study on the conventional and NAT EA detection methods, several EDSV specific PCR assays were tested, and the detection limits of the previously described (2, 3), commercially available and newly developed methods were determined by using dilution series of virus stock solution with known 50% egg infectious dose (EID_{50}) value. PCR assays were performed as recommended by the authors / manufacturers or as it was optimized in our laboratory. The specificity, precision and sensitivity of the best performing gel-based PCR assay (2) were investigated and the method was validated according to the recommendations of OIE and ICH (4, 5).

In the study selected mono- and bivalent vaccine preparations representing different production systems were experimentally infected with known amounts of EDSV and the detection limits of the conventional and PCR detection assays were determined and compared. Samples taken immediately after spiking with a dilution series of EDSV strain B8/78 and at the end of each passage (I-IV) were tested simultaneously for the presence of EDSV by ELISA (conventional test) and PCR.

In the conventional EA test monolayers of chicken embryo liver cells are prepared from the tissues of 14- to 16-day-old embryos, and inoculated with test vaccine, a suitable strain of EDS virus (positive control), while non-inoculated monolayers are maintained as negative control. Cells are incubated for a total of at least 21 days, and subcultured every 4-5 days. Cell cultures are monitored for any signs of cytopathic effect throughout the entire incubation period and finally tested by ELISA specific of EDSV.

In the PCR-based EA test nucleic acid was extracted from the samples by using the QIAamp DNA Mini Kit (Qiagen, Germany) as recommended by the manufacturer and DNA was added to the PCR (2) as template. The reaction was based on the amplification of a 237 bp sequence from the hexon gene by using forward primer 5'- CAC ACA ACT GCA TCT GAC TG -3' and reverse primer 5'- TTG GCG TCT TCA AGG CAC TG -3'.

Results

Following the selection of the best performing PCR assay the method was further optimized and validated. The validation procedure involved the thorough investigation of the different aspects of precision: (i) Repeatability (intra-assay precision) that expresses the precision under the same operating conditions over a short time interval was assessed by testing one series of samples under the same experimental conditions (on 6 different days, by the same operators, in the same thermocycler). (ii) Intermediate precision expresses within-laboratories variations and it was determined by analyzing the same series of samples by two different operators (on 6 different days, in the same thermocycler) (iii) Reproducibility that shows the precision between laboratories was examined in 2 laboratories (R&D and Quality Control) on 6 different days, by 2 operators using different types of thermocyclers. The sensitivity of the reaction that was determined by using serial 10-fold dilutions of EDSV stock solutions (strain B8/78, titer: $10^{+8.4}$ EID_{50}/ml) was approximately $10^{+1.0}$ EID_{50}/ml. The specificity of primers was tested by performing a GenBank BLAST search to exclude the possibility of false-positive results with heterologous avian pathogens (including other adenoviruses) and other viral/bacterial pathogens.

In the comparative assay, where experimentally infected vaccine samples were tested for the presence of EDSV by the conventional EA test and NAT, the validated PCR method was able to detect the virus from the $x10^5$ dilution without any incubation of the samples, whereas the detection limit of the combined cell culture - ELISA detection method was at the $x10^3$ dilution after the 21 days of incubation and passages I-IV.

Discussion and conclusions

The selected gel-based PCR provides a labour- and time-saving, but also a reliable tool for the rapid detection of EDSV in vaccine products. The NAT assay was 100-1000x more sensitive than the *in vivo* EA test and it made the 21-day-long incubation period unnecessary. The replacement of conventional EA tests for NAT would allow us to reduce production costs, the interval between vaccine production and release, and last, but not least, the number of live animal experiments. However, to achieve this goal, validated PCR assays with appropriate sensitivity, specificity and precision values should be available for each *in vivo* EA tests currently in use.

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EARLY PROTECTION AGAINST LETHAL CSFV CHALLENGE INFECTION AFTER IMMUNIZATION OF PIGS WITH THE MODIFIED LIVE MARKER VACCINE CANDIDATE CP7_E2ALF

Ilona REIMANN[1], Immanuel LEIFER[1], Elke LANGE[2], Sandra BLOME[1], Sandra JUANOLA[3], Joan Plana DURAN[3], Martin BEER[1]

[1]Institute of Diagnostic Virology, Friedrich-Loeffler-Institut,Greifswald-Insel Riems, Germany;
[2]Institut of Infectiology, Friedrich-Loeffler-Institut,Greifswald-Insel Riems, Germany;
[3] Fort Dodge Veterinaria SA, Vall de Bianya, Spain

Keywords: Classical swine fever virus (CSFV), candidate marker vaccine, emergency vaccination

Introduction and Objectives

Due to the vast economic consequences of classical swine fever outbreaks, emergency vaccination plans are under discussion in European Union Member States. However, animals vaccinated with the conventional C-strain vaccine are subject to trade restrictions. To ease these restrictions, potent marker vaccines are required. One promising candidate is the chimeric pestivirus CP7_E2alf (3). For emergency vaccination in a CSFV outbreak scenario, rapid onset of immunity is essential. Here, we decribe the outcome of animal experiments designed to further evaluate and compare the modified live prototype vaccine CP7_E2alf, which was produced under good manufacturing conditions (GMP) with the conventional C-strain vaccine. Emphasis was put on the onset of immunity after oral and intramuscular vaccination. In addition, the genetic stability was tested after multiple passaging in cell culture.

Materials and methods

CP7_E2alf virus was produced under GMP conditions (Fort Dodge Veterinaria SA, Vall de Bianya, Spain). Sequencing of the stock virus was carried out using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Nucleotide sequences were read with the 3130 Genetic Analyzer (Applied Biosystems) and subsequently analyzed using the Genetics Computer Group software version 11.0 (Accelrys Inc) and compared with the sequence data of the prototype vaccine stock (11th passage) .

Two animal experiments were done. In the first experimental setup, four 7-9 week old pigs were intramuscularly vaccinated with 2 ml of either CP7_E2alf (2 x $10^{6.0}$ $TCID_{50}$/ml), or C-strain vaccine (2 x $10^{5.25}$ $TCID_{50}$/ml), respectively. One week after vaccination, challenge infection was carried out by oronasal application of 2 ml blood preparation containing CSFV strain Koslov (2 x $10^{6.5}$ $TCID_{50}$/ml). Blood samples and nasal swabs were collected at day zero (day of vaccination), at challenge infection (seven days post vaccination) until day 28 post infection.

In the second experimental setup, four 7-9 week old pigs were oronasally vaccinated with 2 ml of either CP7_E2alf (2 x $10^{6.0}$ $TCID_{50}$/ml), or C-strain vaccine (2 x $10^{5.25}$ $TCID_{50}$/ml), respectively. This time, two weeks after vaccination challenge infection was done. Rectal body temperature and clinical signs were monitored and recorded daily. Blood samples and nasal swabs were collected after immunization as well as after challenge infection and processed for virological and serological testing.

Results

The sequence data of the GMP-produced CP7_E2alf confirmed the sequence of the prototype vaccine stock in general. Nine observed nucleotide substitutions resulted in eight amino acid exchanges but only one of them was detected within the E2 protein, the main antigen of pestiviruses. In addition, an insertion of one nucleotide within the 5'NTR could be detected.

In all intramuscularly vaccinated animals, body temperature stayed within the physiological range, and none of the animals showed clinical signs of CSF following vaccination or challenge infection. Vaccine or challenge virus could neither be isolated from nasal swabs nor from leukocytes and the highly sensitive real-time RT-PCR revealed negative results. All animals seroconverted and glycoprotein E2 specific as well as neutralizing antibodies were detected in all animals two weeks after infection. Compared with C-strain vaccination a very similar antibody response, protection and safety could be observed.

After oral vaccination with CP7_E2alf the pigs again showed full protection in challenge experiments with CSFV strain Koslov. Already one week after infection, E2 specific antibody ELISAs were positive and neutralizing antibodies against CSFV strain Alfort were detected. No virus could be isolated from nasal swabs or leukocytes. Nevertheless, the CSFV genome load after challenge was little higher in the CP7_E2alf group than in the C-strain vaccinated group.

Discussion and conclusions

Our results confirm previous experimental data with early passages of a non-GMP CP7_E2alf vaccine virus (11th passage) and challenge infection on later time point (2, 3) on safety, protection and efficiency. The findings also affirm that CP7_E2alf vaccination of pigs results in full protection against CSFV infection after a very short time period, both after oral and intramuscular vaccination, which is a prerequisite for the use as emergency vaccine (1). In all aspects, CP7_E2alf is comparable with the "gold standard", the C-strain vaccine. Finally, even after several passages and high titer production in a bioreactor only a few mutations could be detected, which might be adaptive for cell culture growth and which did not influence any in vivo properties in our animal trials. Further studies on serial passages will give additional knowledge on the sequence stability. Thus, CP7_E2alf is an auspicious marker vaccine candidate for both oral vaccination of wild boar and emergency vaccination of domestic pigs.

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COEXPRESSION OF RABIES VIRUS GLYCOPROTEIN AND NUCLEOPROTEIN IN SILKWORM-BACULOVIRUS EXPRESSION SYSTEM AND ITS UTILIZATION AS A SUBUNIT VACCINE

Xiangping YIN[1], Zhiyong LI[1], Yinv LI[2] Jiangtao LI[1], Yongzhu YI[2], Huiguo SHU[2], Zhifang ZHANG[2],Jixing LIU[1]

[1]State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Animal Virology of Ministry of Agriculture, Lanzhou Veterinary Research Institute, Chinese Academy of Agriculture Sciences, 1 Xujiaping, Lanzhou, China;
[2]Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, 12 Zhongguancun South Street, Beijing, China

Keywords: rabies virus, glycoprotein, nucleoprotein, Silkworm-baculovirus expression system, vaccine

Introduction and Objectives
Rabies is a fatal zoonotic infectious disease caused by rabies virus, being epidemic worldwide, especially in most Asian and African countries. Even though there are effective vaccines available, eradication of the rabies virus has remained problematic by the high cost of production and administration of the vaccines. Thus, There is a signiticant need for development of effective, safe, and economic rabies vaccines for animals.

Materials and methods
The gene encoding rabies virus glycoprotein and nucleoprotein were modified by site-directed mutagenesis and linked by IRES sequence, then subcloned into baculovirus plasmid transfer vectors pVL1393, and inserted via homologous recombinations into baculovirus *Bombyx mori* nuclear polyhedrosis virus(rBmNPV). Expression of G and N proteins were detected both by immunofluorescence assays (IFAT) with recombinants virus that infected Bm-N cells and by western-blotting. Early fifth-instar silkworms were infected with the recombinant virus and silkworm haemolymph were obtained and detected by rabies virus antigen ELISA kit. Silkworm haemolymph was lysed ultrasonically and cell debris was removed by centrifugation. The suitable supernatant was used to prepare vaccine. Four to five-week-old BALB/c mice were immunized by intramuscular injection at hind limbs or orally perfusion. Rabies virus neutralizing antibodies were determined by the rapid flourescent focus inhibition test. Vaccinated mice were challenged with determined lethal doses of CVS rabies virus.

Results
The expression of glycoprotein and nucleoprotein in Silkworm haemolymph was analyzed by sandwich-ELISA. The expression yield was about 60 fold more than the positive control, but was not detectable in the negative control. Before the challenge, the VNA of 10 injection vaccinated and randomly selected mice was assayed to be above 0.5 IU/ml while no VNA in 10 negative control mice could be detected. The high specific IgA antibody could be detected in orally administrated animals. Both of intramuscularly vaccinated mice and orally vaccinated mice survived after the CVS-24 challenge,while 9 out of 10 unvaccinated control mice died of the infection.

Discussion and conclusions
In this report, A rabies virus subunit vaccine consisting of the viral G protein and N protein was prepared from a Silkworm-baculovirus expression system. The G antigen and N antigen produced in BmN cells and silkworm haemolymph appeared to be authentic based on Western blotting. Our results showed that both intramuscularly and oral administration in mice conferred a protective immune response. I t is feasible to use this vaccine for preventing rabies virus infection in animals.

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IMMUNOCAPTURE ELISA AND RT-PCR IN THE DIAGNOSIS OF PPRV INFECTION

Harun ALBAYRAK[1], Feray ALKAN[2]

[1]Veterinary Control and Research Institute, Samsun, Turkey;
[2]Ankara University, Faculty of Veterinary Medicine, Virology Department, Ankara,Turkey

Keywords: I-ELISA, PPRV, RT-PCR, sheep

Introduction and Objectives

Peste des petits ruminants (PPR) is an important viral disease of goats and sheep prevalent in West Africa and the Middle East. Morbidity and mortality rates vary but can be as high as 100 and 90%, respectively. The clinical signs of this disease may include an erosive stomatitis, catarrhal inflammation of the ocular and nasal mucous membranes and diarrhoea or dysentery (2,3,4,11). Aetiological agent (PPRV) is a member of the morbilli virus genus, which also includes measles, canine distemper, rinderpest virus and viruses of marine mammals, in the family *Paramyxoviridae* (4,5).

Virus isolation and AGID technique are widely used for the diagnosis of PPRV infection using samples from goats and sheep suspected PPRV infection (2,10,11). Because there is a need for a rapid, sensitive and spesific virus detection test for the diagnosis of PPRV infection, new two methods, immuncapture-ELISA (1,7,9) and RT-PCR (8) have been described, recently. It is known that the main advantages of the I-ELISA are rapidity, specificity, no need for sterile test sera and simplicity (9).

In this presentation was aimed to compare the sensitivity of I- ELISA and RT-PCR in the diagnosis of infection using tissue samples from sheep suspected PPRV infection in Turkey and to investigate of the diagnostic value of the frozen tissue samples for RT-PCR.

Materials and methods

One hundred and six samples including lung(n=40), spleen(n=32) , liver (n=10), lymph node(n=3), leukocyte(n=21) from 56 sheep which showed severe lacrimation, nasal discharge, coughing, sign of bronchopneumonia, and diarrhea were used (Figure 1). All of them were tested by using I-ELISA, produced by Pirbright Research Institute and performed as described by Libeau et al.,1994 (9). Out of 106 tissue samples, 26 from 12 sheep were immediately tested by RT-PCR following to the sampling. The other tissue samples (n=80) stored at -80℃ for approximately 3 months were thawed and then tested by RT-PCR.

Virus RNA was extracted using acid guanidium-phenol-chloroform-isoamyl alcohol mixture as described by Chomczynski and Sacchi, 1982 (6). A PPR-spesific primer set selected from the F protein-coding gene sequence were used in this study. The primers PPRVF1b (5'- *AGT ACA AAA GAT TGC TGA TCA CAG T*-3') and PPRVF2d (5'-*GGG TCT CGA AGG CTA GGC CCG AAT A*-3') have been described previously (8). Polymerase chain reaction amplication was performed as described elsewhere (8) with minor modifications (3).

Results

Results of I-ELISA were showed that out of 52 sheep tested, 18 (34.6%) were found to be positive PPRV infection. The positivity rate was estimated as 37.7% (40/106) in tested tissue samples.

In RT-PCR, amplicons of the expected sizes (448 bp) were obtained from samples and reference virus (Nigeria 75/1).

Results of I-ELISA and RT-PCR applied in 26 fresh tissue samples from 12 sheep were showed in Table 1. Out of 12 sheep, 4 (33.3%) and 7(58.3%) were found to be positive for PPRV infection by I-ELISA and RT-PCR, respectively. The positivity rates of tested tissue samples were estimated as %26,9(7/26), %46,1(12/26) for I-ELISA and RT-PCR, respectively.

In 12 sheep tested, there was a total agreement of results (+ or -) in 9 sheep (75.0%) and disagrement in the results with the other 3 sheep (Table 1). The results of I-ELISA and RT-PCR for the different tissues samples were shown in Table 1. Diagnostic value of lung samples was detected as higher than the others. Briefly, we postulated that RT-PCR is sensitive then ELISA when they were used in the fresh tissue samples for the diagnosis of PPRV infection in fresh samples.

Table 1. Results of I-ELISA and RT-PCR in fresh tissue samples

	Number tested	I-ELISA	RT-PCR	I-ELISA+ RT-PCR+	I-ELISA- RT-PCR+	I-ELISA+ RT-PCR -	I-ELISA- RT-PCR -	I-ELISA and/or RT-PCR (+)
Sheep	12	4 (33,3)*	7 (58.3)	4	3	-	5	7
Lung	*12*	*4* (33,3)	*7* (58.3)	*4*	*3*	-	*5*	*7*
Spleen	*10*	*2* (20.0)	*3* (30)	*2*	*1*	-	*7*	*3*
Liver	*1*	-	-	-	*1*	-	*1*	
Lymph nodule	*3*	*1* (33,3)	*1* (33,3)	*1*	-	-	*2*	*1*

( ) * positivity rates

According to the RT-PCR results from 80 frozen-thawed samples (lung, spleen, lymph nodule and, blood) collected from 40 sheep, positivity rate were estimated as 20% (8/40) and %13,75 (11/80) on the basis of sheep and tissue samples, respectively. However 35% (14/40) and 40% (32/80) of sheep and tissue samples had been detected as positive for PPRV antigens by I-ELISA (Table 2).

Table 2. Results of I-ELISA in fresh samples and RT-PCR in frozen-thawed samples

	No of tested	I-ELISA	RT-PCR	I-ELISA+ RT-PCR +	I-ELISA- RT-PCR +	I-ELISA+ RT-PCR -	I-ELISA- RT-PCR -
Sheep	40	14 (35.0)*	8 (20.0)	*6*	*2*	*8*	*24*
Lung	*28*	*11* (39.2)	*1* (3.5)	*1*	-	*10*	*17*
Spleen	*22*	*10* (45.4)	*6* (27.2)	*4*	*2*	*6*	*10*
Lymph nodule	*9*	*6* (66.6)	*4* (44.4)	*3*	*1*	*3*	*2*
Leucocyt	*21*	*5* (23.8)	- -	-	-	*5*	*16*

( )* Positivity rates

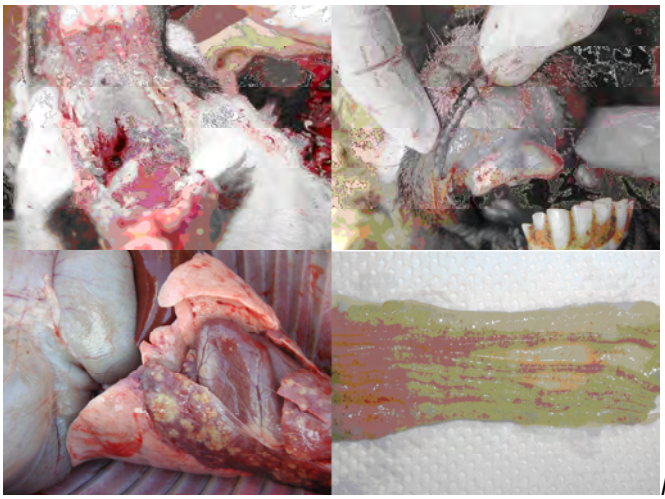

Figure 1. a) Pseudomembranes in the hard palate, b) Erosions in the hard palate and tongue, c) Hepatization area in the lung, d) Zebra line-like bleeding in rectum

Discussion and conclusions

Data showed that the RT-PCR technique permitted the detection of PPR in different fresh samples, which was not possible using ELISA and the diagnostic value of lung samples were the highest for both technique. It is known that quality of sample is very important for test sensitivity of RT-PCR and that especially RNA viruses which are high sequence variability found difficult to design primers for reverse transcription – polymerase chain reaction(7,8). We detected that RT-PCR in frozen samples failed to detect the PPRV nucleic acid in 65% (21/32) of PPRV positive tissue samples by ELISAused in fresh samples. But Diop et al, 2005 (7) reported that even if the cold chain is compromised during sample collection and submission, samples may still be suitable for testing by ELISA and spesific viral antigen detected.

ELISA has been routinely used in reference diagnostic laboratory for the diagnosis of PPRV infection in Turkey. In the present study, the sensitivity of ELISA was lower than RT-PCR for the diagnosis of the infection using fresh tissue samples. It is known that it is essential to use a simple, rapid, sensitive and specific technique in diagnosis of infection. As a result, if I-ELISA is used for the diagnosis of PPRV infection, a plenty of samples must be obtained from flock suspected PPRV infection and/or samples detected as negative by I-ELISA should be tested by RT-PCR for the safety diagnosis.

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DETECTION OF *HEPATITIS E VIRUS*-SPECIFIC IGG ANTIBODIES IN DOMESTIC PIGS FROM GERMANY USING A NOVEL IN-HOUSE ELISA

Christine BÄCHLEIN[1], Anika SCHIELKE[2], Reimar JOHNE[2], Beatrice GRUMMER[1]

[1]Institute of Virology, University of Veterinary Medicine Hannover
[2]Federal Institute for Risk Assessment, Berlin

Keywords: antibodies, Germany, hepatitis E virus, pigs,

Introduction and objectives
Hepatitis E Virus (HEV) is a non-enveloped virus with a positive-sense single stranded RNA genome with three open reading frames (ORFs). In developing countries HEV causes endemic outbreaks of acute hepatitis. In industrialized countries autochthonous infections are increasingly recognized, which could be attributed to the consumption of raw or undercooked meat or liver of pigs or wild boar (Purcell and Emerson, 2008). Furthermore, antibodies against HEV have been detected in healthy blood donors' sera and especially in sera from persons of occupational groups with close contact to pigs, which leads to the assumption of an animal reservoir for HEV (Meng et al., 2002). The objective of this study was to investigate whether HEV is present in the domestic pig population in Germany as it has already been proven for wild boar (Kaci et al., 2008).

Materials and methods
Sera of 1072 pigs randomly collected from ten Federal States were examined by in-house ELISA using ORF2/ORF3 peptides of HEV genotype 1 as antigen (Acris Antibodies GmbH, Germany). Sera were diluted 1:250 in phosphate buffered saline supplemented with one percent horse serum. To raise specificity, a stringent wash with 3 M urea was performed after incubation of testing sera. Polyclonal rabbit anti pig-IgG (Sigma-Aldrich, Germany) was used as conjugate. Reference sera from Spanish pigs served as controls for the establishment of the ELISA and for determination of positive/negative cut-off values.

Results
The overall seroprevalence was determined as 49.5 %, ranging from 15.6 % (Mecklenburg-Western Pomerania) to 70.7 % (Bavaria). The intra-assay coefficient of the controls was calculated as 6.2%, and 3.9%, the inter-assay coefficient was determined as 14.5% and 20.2%. Results have been confirmed for reference sera and selected field sera, which were tested in parallel with a commercially available Western blot (Mikrogen, Germany). In addition, 297 field sera have been retested in a commercially available HEV double sandwich antibody ELISA (Axiom, Germany). Comparison of both ELISAs showed a rather low sensitivity and specificity of about 60 %.

Discussion and conclusions
We developed an ELISA detecting porcine anti HEV antibodies. As sequence comparisons revealed, there is a high homology between the genotype 1 derived peptides and the corresponding genotype 3 peptides. Though, it seemed reasonable that we used the genotype 1 peptides although genotype 3 is the only HEV genotype which could be found in European pigs so far.

The serological screening with the new in-house ELISA revealed a high seroprevalence in domestic pigs in Germany which is concordant to similar findings in other European countries (de Deus et al., 2008). The results of our ELISA match a lot more the findings of a commercially available Western blot than those of a purchasable ELISA. Several explanations for these discrepancies are possible: each test uses varying peptides corresponding to amino-acid residues in ORF2 or ORF3. Besides, the novel test demonstrates HEV specific IgG antibodies whereas the Axiom ELISA detects antibodies of the IgG and IgM subtype simultaneously.

Swine HEV infection appears to be widespread in German commercial piggeries and in wild pigs. The assay based on a highly conserved epitope of a human HEV strain is reactive with porcine antibodies. Possible effects of swine HEV on animal production and its' role in human disease remain to be established but are reasons for concern.

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POTENTIAL RISKS FOR THE VERTICAL TRANSMISSION OF BOVINE VIRAL DIARRHOEA (BVD) VIRUS

Jelena BALATINEC, Tomislav KEROS, Mirko LOJKIĆ, Lorena JEMERŠIĆ, Ivana LOJKIĆ, Željko CVETNIĆ, Tomislav BEDEKOVIĆ

Croatian Veterinary Institute, Zagreb, Croatia

Keywords: BVD, PCR, transmission

Introduction and Objectives

The current breeds of beef cattle are selected for their high productivity and capacity for adaptation to farm breeding.In raising beef cattle, any disease transmission to the first filial generation can be prevented by implementing effective prevention and treatment strategies. The paper shows the results obtained in the study of beef cattle samples of the same herd conducted to determine the potential risk and occurrence of bovine viral diarrhoea (BVD) virus in filial calves.

Materials and methods

We tested 21 randomly selected bovine samples (17 bovine blood samples and 4 bull semen samples) from the BVD infected area. Previous studies by comparative analysis of 11 microsatellite loci have shown that the selected animals are interbred. The technique employed includes RNA extraction from bovine blood and bull semen samples using the QIAamp kit. The polymerase chain reaction (PCR) products are then analysed by gel electrophoresis to see whether there are any sequences indicating the presence of BVD virus in any of the study samples.

Results

Since the tested samples of bovine blood and bull semen of the same herd were determined negative for the presence of bovine viral diarrhoea (BVD) virus the assumption of potential infection transmission couldn't be confirmed nor refuted.

Discussion and conclusions

The aim of this paper is to assess the risk of BVD virus transmission from parent to the next 4filial generation. In case there is a potential risk of transmission,the animals should be tested immediately after birth to determine whether they are infected by BDV virus. Since the assumption of potential infection transmission could be neitherconfirmed nor refuted in this study, the need for further research involving a larger number of blood samples, particularly bull semen samples, is underlined.

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DETECTION OF A NOVEL PORCINE BOCA-LIKE VIRUS IN THE BACKGROUND OF PORCINE CIRCOVIRUS TYPE 2 INDUCED POSTWEANING MULTISYSTEMIC WASTING SYNDROME

Anne-Lie BLOMSTRÖM[1], Sandor BELÁK[1,2], Caroline FOSSUM[3], John MCKILLEN[4], Gordon ALLAN[4], Per WALLGREN[2], Mikael BERG[1]

[1]Department of Biomedical Sciences and Veterinary Public Health, Section of Parasitology and Virology, Swedish University of Agricultural Sciences, Uppsala Sweden; [2]National Veterinary Institute, Uppsala, Sweden; [3]Department of Biomedical Sciences and Veterinary Public Health, Section of Immunology, Swedish University of Agricultural Sciences, Uppsala, Sweden; [4]Queens University Belfast and Agri-Food and Biosciences Institute, Belfast, Northern Irland

Keywords: bocavirus, PCV-2, PMWS, random multiple displacement amplification

Introduction and objectives

The causative agent of post weaning multisystemic wasting syndrome (PMWS) is porcine circovirus type 2 (PCV-2). The virus is however considered to be a ubiquitous agent in pigs and most of the pigs with a PCV-2 infection do not develop any disease. Experimental studies have shown that other factors apart from PCV-2 are usually needed in order for the pig to develop the complete complex clinical picture of PMWS. Such factors are co-infection with other viruses such as PRRS and PPV (1). In this study the overall viral DNA environment in two lympnodes from Swedish pigs with PMWS was investigated utilising random multiple displacement amplification (MDA) and large scale sequencing.

Materials and methods

Lymph nodes from two Swedish pig previously confirmed with PMWS was used in this study. Before DNA extraction the samples were pre-treated in order to reduce the host DNA and the DNA was then extracted followed by an MDA amplification. The amplified DNA was sequenced using the 454 technology. The data was analysed through assembly and database searches. PCR was set up for further characterisation and detection of the different viruses found.

Results

Sequencing revealed apart from PCV-2 sequences (99.98 %) also the presence of Torque Teno virus (TTV) (0.107 %). Genotype specific PCR showed the presence of both TTV-1 and TTV-2. We also found sequences from a porcine parvovirus (0.076 %). Designing primers based on the sequences obtained by the 454 run allowed us to amplify a larger region of the found parvovirus; this region was shown to consist of the entire NP1 gene and partial VP1/2. The NP1 gene that is characteristic for bocaviruses had a 33 - 36 % identity to other NP1 genes of bocaviruses (Human bocavirus, Canine minute virus and Bovine parvovirus). Phylogenetic studies based on a region of the VP1/2 revealed that the parvovirus found in this study grouped together with the bocaviruses rather than with previously described porcine parvoviruses (fig.1).

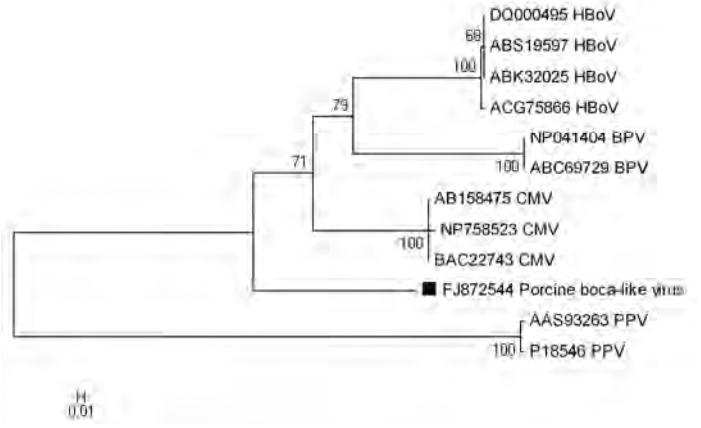

Fig 1: Neighbour joining tree based on a part of the VP1/2 gene. HBoV (Human bocavirus), BPV (Bovine parvovirus), PBo-likeV (Porcine boca-like virus), CMV (Canine minute virus), PPV (Porcine parvovirus).

Discussion and conclusions

A vast majority of the sequences obtained by the 454 run was from PCV-2. Only around 30 out of the 8000 sequences were of other origin. The pre-treatment appeared to have worked well since only one of the sequences were of host origin. Eighteen sequences apart from the found viruses are of unknown origin showing no relationship to any sequences in the Genbank neither on nucleotide nor protein level. This might be due to the short nature of some of these sequences or to the fact that they might be the real presently unknown viruses. The finding of TTV was expected since this virus has in other countries been shown to be as PCV-2 almost ubiquitous. The finding of the porcine boca-like parvovirus was interesting not only because this it a novel porcine bocavirus but also since parvoviruses have been shown to together with PCV-2 be able to invoke the complete clinical picture of PMWS. The co-infection of at least three viruses is interesting considering that there is still quite many un-clear questions concerning the aetiology and disease development of PMWS.

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FIELD EVALUATION OF A BULK MILK MONITORING PROGRAMME OF BLUETONGUE VIRUS 8 (BTV-8) INFECTION IN A BLUETONGUE FREE AREA

Giulio BUCCI[1], Ernst STIFTER[2], Lebana BONFANTI[3], Laura GAGLIAZZO[3], Stefano NARDELLI[1]

[1]Istituto Zooprofilattico Sperimentale delle Venezie, Animal Health Department, Legnaro (PD), Italy;
[2]Provincial Veterinary Service, Autonomous Province of Bolzano, Italy;
[3]Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro (PD), Italy

Keywords: bluetongue, monitoring programme, bulk milk

Introduction and Objective

In 2006, the first outbreak of bluetongue (BT) virus in northern Europe was reported, and the serotype was identified as BT virus 8 (BTV-8). The virus overwintered and reappeared in May-June 2007. In March 2008, the first outbreak of BTV-8 in Italy was reported. At the time, the BT serological monitoring programme in the area of the outbreak used provinces as epidemiological units and required at least 150 heads per province distributed in 10 herds to identify a prevalence of 2% of BT infection with confidence intervals of 95% (2). Sampling was performed once a month, though samples were not collected during the vector free period (from January to February). Taking into account the risk of BTV-8 introduction through the importation of beef cattle mainly from France, a monitoring programme based on the application of a commercial ELISA on bulk milk samples was carried out on a limited number of dairy cattle farms, mainly to evaluate the validity of the diagnostic test.

Materials and Methods

To assess the specificity and analytical sensitivity of the ELISA, a preliminary evaluation was conducted. Bulk milk samples from herds located in areas that at the time had never been affected by BT were tested: 576 in the Bolzano Province and 683 in the Veneto Region.

A second evaluation was conducted to assess the sensitivity of the ELISA. Individual milk samples from cows whose blood samples tested positive were diluted in a BT-tested negative milk, with a initial dilution of 1:12.5 and doubling the dilution until reaching 1:400.

The field evaluation was conducted in the Veneto Region (north-eastern Italy). In the spring of 2008, bulk milk samples from 3,932 cattle herds, out of a total of approximately 16,000 cattle herds in the study area, were collected. Each sample was added a Na-azide (NaN_3) tablet as preservative and the samples were stored at 4° C.

Samples were tested with a commercial Elisa kit (ID.VET Blue Tongue Milk Indirect) designed to detect antibodies against the BT virus vp 7 protein. To improve sensitivity, a cut-off of 25% was used, which is lower than the suggested cut-off. For herds with positive bulk milk samples, blood samples were collected from each bovine and tested with a commercial ELISA kit for group specific antibodies. The ELISA positive serum samples were sent to the National Reference Laboratory for BT (CESME) and were evaluated by virus neutralization (VN) test to identify the BT serotype involved.

Results

The results of the specificity test are reported in Table 1. All of the cows from herds whose bulk milk had tested positive during the specificity evaluation showed negative serological results.

The mean sample/positive (S/P) value for ELISA was higher in the Bolzano Province than in Veneto Region (p=0.018).

Table 1. Specificity assessed on bulk milk samples from cows from areas unaffected by BT

AREA	NUMBER OF HERDS	MEAN S/P VALUE	NUMBER OF LACTATING COWS/HERD	SPECIFICITY
BOLZANO	576	8.18	10	97.9%
VENETO	683	5.50	40	99.4%

Table 2. Analytical Sensitivity assessed on individual positive milk samples

MILK DILUTION	NUMBER OF MILK SAMPLES TESTED POSITIVE	SENSITIVITY
1:12.5	26	100%
1:25	26	100%
1:50	26	100%
1:100	23	88.46%
1:200	18	69.23%
1:400	12	46.15%

The sensitivity was 100% for dilution up to 1:50; for higher dilution it gradually decreased (Table 2).

In Veneto, 8 of the 3,932 herds were positive to the bulk milk ELISA. Three of these herds showed positive blood samples (Table 3). These 3 herds were the 3 BT outbreaks already identified by means of the official monitoring programme.

According to VN, the serotype was BTV-8 in all of the seropositive cows.

Table 3 - Serological testing of cattle herds with positive bulk milk samples

HERD	BLOOD SAMPLES		BT OUTBREAK
	NUMBER OF BOVINE IN THE HERD	NUMBER OF POSITIVE ANIMALS	
1	5	0	NO
2	6	0	NO
3	8	0	NO
4	45	0	NO
5	65	0	NO
6	11	11	YES
7	91	4	YES
8	118	9	YES

Discussion and Conclusion

The results of this study demonstrate that the high sensitivity and specificity of the ELISA BT test applied to individual milk samples (1) are confirmed for bulk milk too. The significant difference in specificity (p=0.018) when comparing Bolzano (with approximately 10 lactating cows per herd) and Veneto (approximately 40 lactating cows per herd) may suggest that bulk milk samples taken from few cows could have higher S/P values, possibly because of the lower dilution of unspecific factors. The difference could also be related to the specific breed: in Veneto the prevalent breed is Holstein, whereas in Bolzano is Braunvieh.

The finding of a high sensitivity suggests that bulk milk sampling may be more effective than serological sampling in identifying positive herds because it allows a larger number of cows to be tested. For example, the probability of detecting 1 BT-seropositive head using bulk milk sampling in a herd of 50 lactating cows would be 100%, whereas the probability would be only 30% if performing blood testing on 15 sentinel animals, as recommended by the National serological surveillance programme. Moreover, the ease with which bulk milk samples can be collected allows a large number of herds to be tested in a short period of time, with markedly less labour and stress for cows.

It should be highlighted that these results are applicable only for recent BTV-8 infection in unvaccinated areas. After 1 year, the antibody titres in bulk milk moderately decrease, but further studies are needed.

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DEVELOPEMENT OF A NOVEL REAL TIME RT-PCR FOR THE DETECTION OF THE NS2A GENE OF WEST NILE VIRUS (WNV)

Antonella CERSINI[1], Ilaria Maria CIABATTI[1], Armando DAMIANI[2], Giuseppe MANNA[1], Maria Teresa SCICLUNA[1], Gian Luca AUTORINO[1]

[1]Istituto Zooprofilattico Sperimentale Lazio e Toscana, Roma, Italy;
[2]Department of Veterinary Microbiology and Immunology, Cornell University,USA

Keywords: NS2a gene, Real-time RT-PCR, West Nile virus

Introduction and objectives

WNV belongs to the family of Flaviviridae, genus Flavivirus and is grouped into the Japanese Encephalitis antigen complex. Four genetic lineages with specific geographic distributions have been described. The Real Time RT-PCRs reported in literature, targeted to the 3' non coding (NC) region, to the E and the NS5 genes, are mainly specific to strains belonging to lineage 1. Recently lineage 2 was detected in both the Mediterranean area as well as in northern Europe, therefore our group proceeded with the development of a novel Real-time RT-PCR for the detection of the aforementioned lineages.

Moreover, an in vitro transcribed RNA, specific for target NS2a (mRNA NS2a) has been synthetized to estimate the efficiency of the method and also as a non hazardous positive control in diagnostic routine.

Materials and methods

Negative samples prepared for the devlopment of the method were derived from the spinal chord of a regularly slaughtered horse, tested negative for the gene NC of WNV. While the postive samples were obtained seeding the same material with refernce strain Egypt 101.

Total RNA extraction was carried out using Fast RNA® Pro Green Kit (Q-BIO gene) and products were purified using silicon-based columns (Qiagen), following the manufacturer's instructions.

Primers and Taq-man probes were designed on the multialignment of the different genomes of virus available in GenBank using Clustal W method, employing Lasergene programme (DNA Star Inc., versione 5, Madison WI, USA).

The prime and probe targets was selected in a highly conserved genomic region of the WNV, 46 nucleotides long, present in the NS1 and continuing into the NS2a, for 133 nucleotides. The consensus sequence was analysed using the Primer Express version 3,0 programme (Applied Biosystems) revealing a target extending from the 3516° to the 3621° nucleotide. The primer sequence was confirmed using GenBank BLAST. Optimization of the Real-time RT-PCR was obtained verifying different concentrations of primers and probe using the viral RNA extracted from Egypt 101 grown in cells, as well as the transcribed RNA, using ABI PRISM 7900 HT(Applied Biosystems).

The in vitro transcribed RNA was synthetized using plasmide pCRII-TOPO (TOPO TA Cloning® Kit Dual Promoter, Invitrogen). The clone sequence was confirmed by BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) using ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). The in vitro transcription reaction was carried out employing kit Mega script T7/Sp6 (Ambion).

The evaluation of the sensitivity of the method was compared with that targeting the NC region (1). The specificity of the assay was tested using the WNV lineage 1 strains, NY99-snowy owl (ATCC), NY99 equine, NY99 crow, Arb 310/67, Egypt 101, Italy 98, of American, Central African, North African and European origin respectively: WNV Lineage 2, B956(ATCC) strain from Uganda: Usutu virus, strain SAR 1776 of South Africana origin: Border Disease virus (BDV) and Equine herpesvirus type 1 (EHV-1), both responsible for encephalomyelitis infections in horses. For the relative sensitivity and the elaboration of a standard reference curve, successive log $_{10}$ serial dilutions of cell-extracted total RNA were used.

Results

For the the standard curve, a linear relationship between the target dilutions and the Ct was observed with a R^2 Correlation Coefficient value of 0.99, indicating an optimal performance of the selected primers.

A 90% test efficiency was observed, using the formula E=10(-1/slope)–1, employing the in vitro NS2a RNA, while the linearity assay was between 106 molecoles (Ct=20) and about 120 molecoles (Ct = 33).

The analytical sensitivity of our method was comparable to the NC Real -time RT-PCR while the diagnostic sensitivity was superior to the latter, detecting the Uganda B956 strain, belonging to lineage 2.

Discussion and conclusions

The selected primers and probes resulted specific for WNV, not having amplified either correlated virus (Usutu virus), or regions of viruses also causing encephalomielatis in horses (BDV, EHV-1). Further more the assay detected a WNV lineage 2, for which our area, the Meditarrean basin, is more at risk of introduction, due to the bird migratory routes. Finally, considering the zoonotic aspect of the infection, the production of an in vitro transcribed RNA, specific for target NS2a minimises the biological risk of this virus in the laboratory.

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PHYLOGENETIC ANALYSIS OF THE HEXON GENE OF AVIAN ADENOVIRUS FROM COMMERCIAL CHICKENS AND TURKEYS

Jorge L. CHACÓN[1], Antonio C. PEDROSO[1], Juliana RODRIGUEZ[2], Camila PELOSO[1], Joelma MOURA[1], Claudete FERREIRA[1], Antonio P. FERREIRA[1]

[1]Laboratory of Avian Pathology, University of São Paulo, São Paulo, Brazil;
[2]Institute of Biomedic Science, University of São Paulo, São Paulo, Brazil

Keywords: Avian adenovirus. Phylogenetic analysis. PCR. Chicken. Turkey.

Introduction and Objectives

The *Adenoviridae* family includes *Mastadenovirus*, *Siadenovirus*, *Atadenovirus*, and *Aviadenovirus* genera. The *Aviadenovirus* genus, formerly designated as group I Avian adenoviruses (AAV), contains 11 of the 12 recognized European adenovirus serotypes classified in five (A to E) molecular groups. AAV have been associated with inclusion body hepatitis and hydropericardium syndrome, respiratory disease, necrotizing pancreatitis, and gizzard erosions. Different serotypes, and even strains of the same serotype, can vary in their ability to produce illness and death. The objective of this study was to characterize AAV detected from Brazilian commercial chicken and turkey flocks, which showed depression, diarrhea, enteric hemorrhagic, growth retardation, increased feed conversion rate and mortality

Materials and methods

Samples: Between 2004 and 2008, two broiler farms and two Turkey flocks from three Brazilian States reported outbreaks of moderate-to-severe enteric disease. Samples of enteric contents were taken as pools of five birds after necropsy. The samples were sent to the Laboratory under refrigeration. The samples were prepared as 20% w/v suspensions in PBS 0.01 M pH 7.4 and clarified at 1,500 x g for 15 minutes, the supernatants being collected for analysis. Then, DNA was extracted according to Chomczynski (1993).

Adenovirus detection: Detection of avian was performed by a PCR aimed to amplify a 987-bp fragment of the AAV hexon gene. Primers and PCR conditions described by Alvarado et al. (1997) were used.

DNA sequencing and Phylogenetic analysis: The PCR products of the hexon gene from four samples were purified and submitted to DNA sequencing. The homology of the sequences from AAV obtained was evaluated by alignment and comparison using BLASTn. To determine relationships among AAV samples, phylogenetic analysis of the aligned sequences was performed. A Neighbor-joining distance tree with Kimura-2- parameter model was built with Mega 4.1. The Brazilian isolates detected and sequenced in this work were aligned with previously published sequences in Genbank.

Results

Two samples from turkey flocks and two samples from chicken flocks were positive by PCR. Then, the PCR products were purified and submitted to DNA sequencing to confirm the specificity of the amplicons. The DNA sequencing confirmed the PCR results.

Table 1. Nucleotide similarities of the hexon gene among Brazilian turkey and Fowl AAV and other AAV.

Seq->	1 CELO	2 SR48	3 75	4 506	5 340	6 CR119	7 ATCC	8 8565	8 TR59	9 764	9 Stanf	10 ATCC	11 C2B	11 1047	12 380	TAdV USP	TAdV USP	FAdV USP	FAdV USP
FAdV 1 strain CELO	64.4	64.8	68.1	66.2	66.6	67.5	66.5	66.6	65.9	66.2	65.5	64.6	64.4	64.4	71.3	71.3	64.6	63.9	
FAdV 2 strain SR48		79.7	61.4	69	75.2	73.6	74.4	74.1	72	71.8	72.2	98.9	96.7	98.8	64.3	64.3	97.8	94.1	
FAdV 3 strain 75			60.1	68.1	72.6	71.8	71.3	70.9	71.1	70.9	70.9	80.1	78.5	79.7	64.1	64.1	79.2	77.3	
FAdV 4 strain 506				62.8	62.4	63.8	63.9	64	61.5	61.8	61.6	61	61.4	61	71.2	71.2	61.3	61.4	
FAdV 5 strain 340					71.6	71.3	71.8	71.3	69.5	69.5	69.6	69	68.7	68.7	66.8	66.8	69.1	68.6	
FAdV 6 strain CR119						88.2	86	85.5	84.2	83.6	84.8	74.5	73.7	74.4	66	66	74.2	74.6	
FAdV 7 ATCC VR-832							86	85.6	90.2	89.5	90.2	73.1	72.4	73.1	66.7	66.7	73.1	73.2	
FAdV 8 isolate 8565								99.5	85.1	85.1	86.1	74.1	73.2	74	67.9	67.9	74	74.1	
FAdV 8 strain TR59									84.8	84.9	85.7	73.9	73	73.7	68.1	68.1	73.7	73.9	
FAdV 9 strain 764										98.7	97.7	71.7	71.1	71.6	65.1	65.1	71.9	72.4	
FAdV 9 isolate Stanford											97.5	71.5	70.9	71.4	65.3	65.3	71.6	72.3	
FAdV 10 ATCC VR-835												72	71.6	71.9	65.5	65.5	72.4	73	
FAdV 11 strain C2B													97.2	99.6	64.1	64.1	97.8	94.1	
FAdV 11 isolate 1047														97.1	64	64	98.6	95.1	
FAdV 12 strain 380															64.1	64.1	97.7	94.1	
TAdV USP-264-1 PL-1																99.2	64.1	63.3	
TAdV USP-264-2 PL-2																	64.1	63.3	
TAdV USP-11 PL-1																		94.7	
TAdV USP-27 PL-1																			

Nucleotide sequences showed 94.7 and 99.2% identity among the chicken and turkey samples, respectively. By phylogenetic analysis, the samples from chicken were more closely related (95.1 and 98.6%) with the European AAV 11 serotype. On the other hand, the samples from turkeys exhibited low similarity (63.3 to 71.3%) with reference strains belonging to the 11 AAV serotypes.

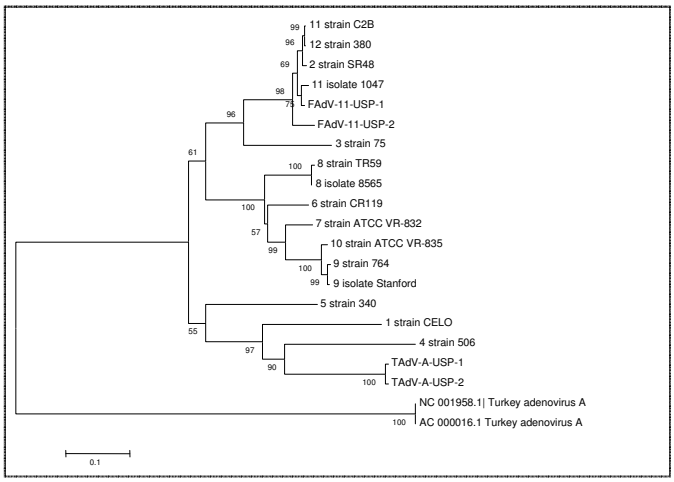

Figure 1. Phylogenetic relationships among AAV. Following alignment of sequences from the hexon gene.

Discussion and conclusions

According the phylogenetic analysis, the Brazilian FAdVs belong to serotype 11 and the Brazilian TAdVs formed a different phylogenetic group to reported FAdV and TAdV from group I. In contrast with the clear association of subgroup II (turkey hemorrhagic enteritis and related viruses, and subgroup III (egg drop syndrome) adenoviruses with disease, the role of most subgroup I avian adenoviruses as pathogens is not well defined. Exceptions include the FAdV-1 strains, which cause Quail Bronchitis and also the FAdV-4 strains, which play a major role in the etiology of hydropericardium syndrome. In addition, other strains rapidly can exploit opportunities presented when the health of the bird is compromised (e.g., by co-infection with other pathogens such as chicken infectious anemia virus [CIAV] or infectious bursal disease virus [IBDV]). Consequently, studies are required to know the pathogenic and phylogenic relationships between Turkey and chicken AAV. The Avian adenoviruses detected in this study were found in commercial birds with severe enteric disturbs, however, experimental challenges are necessaries to know the degree of virulent of these viruses. We reported for the first time Avian adenovirus presents in Brazilian commercial flocks.

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EVALUATION OF THE CACO-2 CELL LINE FOR ISOLATION OF SWINE INFLUENZA VIRUS COMPARED TO STANDARD METHODS

CHIARA CHIAPPONI, IRENE ZANNI, CHIARA GARBARINO, GIUSEPPE BARIGAZZI AND EMANUELA FONI

Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini", Sezione Diagnostica di Parma, via dei Mercati 13/A, 43100 Parma Italy

Keywords: swine influenza virus, CACO-2, MDCK, embryonated chicken eggs, isolation

Introduction and Objectives

Swine influenza viruses (SIVs) circulating in Italy and Europe originated from the genetic reassortment of avian and human strains. In Europe three main influenza A subtypes (H1N1, H1N2, H3N2) circulate in swine populations. A continuous evolution of reassortant viruses is observed. Moreover recently a novel swine-like human influenza A/H1N1 virus (1) demonstrated to be able to cross species barrier causing infection in a pig herd in Canada (2). Viral isolation and identification procedures are time-consuming methods but are necessary steps when antigenic and genetic studies, critical for epidemiology and particularly for monitoring of risks of virus transmission among animal species, are required. Isolation of influenza viruses in embryonated chicken eggs (ECE) is considered the "gold standard" method. Nevertheless cell lines have shown to be a reliable system. Influenza A viruses have different host in vitro growth properties depending on the strain and the preferential cell receptor involved in cell infection. Recently a human colon intestinal epithelium cell line (CACO-2) was shown to be efficient, without adding trypsin in the medium, for isolation of H3N2 and H1N1 human influenza viruses from clinical samples (3). The objective of this study was to evaluate the use of the CACO-2 cell line for the isolation of swine influenza viruses H1N1, H1N2, H3N2 from clinical samples in comparison to the MDCK cell line and ECE inoculation.

Materials and methods

A swine influenza virus monitoring program was carried out from 2006 to 2008 involving 132 pigs farms located in a high density swine area in Northern Italy. Forty-two outbreaks of respiratory disease caused by influenza virus were diagnosed by RT-PCR on pooled samples from lungs and nasal swabs. One hundred and four samples from positive pools were selected and tested for virus isolation on the three substrates at the same time. Tissue samples and swabs were homogenized in PBS with antibiotics and were inoculated in 10 day old specific pathogen free ECE. MDCK cells were grown in 24-well plates, 0.2 ml of filtered homogenate sample was inoculated onto 5 wells and 0.2 ml of MEM containing trypsin (10µg/ml) was added. CACO-2 cells were propagated in 24 well plates in Eagle's minimum essential medium in Earle's BSS with 1 mM of sodium pyruvate, 0.2 ml of filtered homogenate sample was inoculated onto 5 wells of CACO-2 cells and 0.2 ml of MEM with 1 mM of sodium pyruvate was added . Allantoic fluids and supernatants of infected cells were collected and tested for SIV presence by hemagglutination test and by ELISA test for nucleoprotein A. Samples resulted negative for influenza virus had a second passage. Influenza virus positive samples were characterized using reference antisera by hemagglutination inhibition test and by multiplex RT-PCR for HA and NA subtyping. Results were analysed using the Fisher's exact test.

Results

Sixty viruses (16 H1N1, 28 H1N2 and 16 H3N2) from 42 different respiratory outbreaks were isolated. All the H1N1 (16/16) and 28/28 H1N2 SIVs were isolated using the CACO-2 cells even if a serial passage was requested in 50% of the cases. In particular 24/28 H1N2 were able to replicate in the CACO-2 cells only, demonstrating a statistically significant difference (Fisher's exact test, $p<0.01$) with respect to the MDCK cell line and to ECE for H1N1 and H1N2 subtype isolation.

In ECE 7/16 H1N1, 3/28 H1N2 and 16/16 H3N2 were isolated, and almost all viruses isolated were detected at the first passage. ECE inoculation allowed more H3N2 viruses to be isolated than the cell culture systems (Fisher's exact test, $p<0.01$). MDCK cells displayed a low virus recovery rate compared with the other two systems i.e. 9/16 H1N1, 1/28 H1N2 and 6/16 H3N2 (table1).Table 2 shows the percentage rates of SIV isolation using the three growth systems, evaluated alone or combined. One hundred percent of H1N1 and H1N2 subtypes were isolated using CACO-2 cells alone, while isolation of the H3N2 subtype occurred in 50% of the cases. Contemporary use of ECE and CACO-2 cells allowed the isolation of 100% of SIVs involved in the outbreaks.

Table 1. Number of SIV isolates in ECE, CACO-2 cells and MDCK cells. (I)first passage. (II)second passage.

	ECE I	ECE II	CACO-2 I	CACO-2 II	MDCK I	MDCK II
H1N2	3	0	13	15	0	1
	3		28		1	
H1N1	6	1	8	8	4	5
	7		16		9	
H3N2	15	1	7	1	6	0
	16		8		6	

Table 2. Isolation rate of H1N1, H1N2, H3N2 viruses with the different culture systems, either alone (a-c) or combined (d-g).

	(a) CACO-2	(b) MDCK	(c) ECE	(d) CACO-2 + MDCK	(e) CACO-2 + ECE	(f) MDCK + ECE	(g) CACO-2 + MDCK + ECE
H1N1	16/16 (100%)	9/16 (56%)	7/16 (44%)	16/16 (100%)	16/16 (100%)	10/16 (63%)	16/16 (100%)
H1N2	28/28 (100%)	1/28 (3.5%)	3/28 (11%)	28/28 (100%)	28/28 (100%)	4/28 (14%)	28/28 (100%)
H3N2	8/16 (50%)	6/16 (38%)	16/16 (100%)	8/16 (50%)	16/16 (100%)	16/16 (100%)	16/16 (100%)

Discussion and conclusions

CACO-2 cells showed good performance, confirming published data in studies on human influenza viruses (4). We observed a link between hemagglutinin subtype and cellular tropism. H1N1 viruses showed a better isolation ratio in CACO-2 cells than in the other two systems. H1N2 virus isolation was strongly influenced by the use of CACO-2 cells. Eighty-six percent of them were isolated exclusively from CACO-2 cells and the combined use of MDCK and ECE did not improve the isolation rate. H3N2 virus detection was highest using ECE. Due to the poor sensitivity of the CACO-2 cells to H3N2, it is essential to maintain simultaneous use of two systems, namely ECE and CACO-2 cells, to detect all three subtypes involved in swine influenza. Further detailed genetic investigations could add more information and might perhaps explain the different behaviour of SIV subtypes on the various substrates.

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A COMMERCIALLY AVAILABLE IN-HOUSE TEST IS ABLE TO DETECT THE NEW VARIANT 2C OF CANINE PARVOVIRUS

Nicola DECARO[1], Costantina DESARIO[1], Melissa J. BEALL[2], Maria Loredana COLAIANNI[1], Anthony A. DIMARCO[2], Francesca AMORISCO[1], ALESSANDRA CAVALLI[1], Canio BUONAVOGLIA[1]

[1]Department of Veterinary Public Health, University of Bari, Valenzano (Bari), Italy;
[2]IDEXX Laboratories, Inc., Westbrook, Maine, USA

Keywords: canine parvovirus, diagnosis, in-house assay; new variant 2c

Introduction and Objectives

Canine parvovirus (CPV) is one of the main pathogens causing haemorrhagic gastroenteritis and mortality in pups, and despite extensive vaccination it is still widespread worldwide. The original type CPV-2 was first reported in the 1970s, but soon after its emergence it was replaced in the field by two antigenic variants, CPV-2a and CPV-2b (Truyen, 2006). CPV-2 no longer circulates in the field but it is still present in most vaccine formulations against canine parvovirosis, whereas few vaccines are currently available in the market that are prepared with CPV-2b. A third variant, CPV-2c, emerged in Italy in 2000 (Buonavoglia et al., 2001) and suddenly spread to the canine population worldwide (Decaro et al., 2007, 2009). Recently, concerns have been expressed about the ability of the in-house tests to detect the new variant 2c at the same extent of CPV-2a/2b. In this study, we have evaluated for the first time the detection rates of the different CPV variants by using a commercially available in-house test.

Materials and methods

Samples

A total of 201 specimens were tested that included 58 faecal samples and 143 rectal swabs. The samples were recruited from previous studies (Decaro et al., 2007, 2009) and from the routine CPV diagnostic service, considering only those specimens that contained CPV DNA loads higher than 10^5 DNA copies mg^{-1} faeces. The sample distribution according to the virus type was CPV-2a, *n* = 51; CPV-2b, *n* = 50; CPV-2c, *n* = 100, as determined by minor groove binder (MGB) probe assays able to discriminate the variants and differentiate between vaccine and field strains (Decaro et al., 2007). At the geographical level, the samples were mostly from Italy (n = 125), with other specimens from United Kingdom (*n* = 56), Spain (*n* = 5), and Greece (*n* = 15).

In-house test

The in-house test was carried out with the commercial kit SNAP® Canine Parvovirus Antigen Test (IDEXX Laboratories) and all testing was performed blinded to CPV strain and viral load. Performance of the in-house diagnostic test was assessed by calculating the percentage of positive results for each group of samples based on CPV subtype. Confidence intervals about the means were calculated using exact binomial limits with an alpha of 5% (Excel, Microsoft) and statistical significance was determined with the Chi Square test for multiple proportions with an alpha of 0.05.

Virus isolation

Three CPV-2b samples that tested negative by the SNAP Parvo test despite the presence of very high viral DNA loads (10^9-10^{10} DNA copies mg^{-1} faeces) were inoculated on cell cultures, as previously described (Desario et al., 2005), in an attempt to obtain virus isolates to test on the in-house assay.

PCR amplification and sequence analysis

The three high-titre CPV-2b samples that tested negative by SNAP Parvo were subjected to PCR amplification and sequence analysis of the full-length VP2 protein gene using the Takara LA TaqTM kit (Cambrex Bio Science Milano S.r.l., Italy) and three different primer pairs, as previously described (Decaro et al., 2009). The VP2 sequences obtained were compared with those of reference CPV-2b strains available in the GenBank database.

Results

The in-house assay was able to detect 41/51 types 2a, 39/50 types 2b and 77/100 types 2c CPVs, and the percentage of positive in-house tests was 80.4%, 78.0% and 77.0% for CPV types 2a, 2b and 2c The distribution of the CPV samples by SNAP result and viral titre showed that samples with a viral load higher than 10^9 DNA copies/mg faeces were generally detected by the in-house assay with the exception of three CPV-2b samples that gave a negative reaction despite very high CPV loads (10^9-10^{11} DNA copies/mg faeces) calculated by real-time PCR. The three high-titre CPV-2b samples (all from the United Kingdom) were inoculated on A-72 cells and the virus isolation was successful with two strains. The SNAP Parvo test gave positive results with both viral isolates. The full-length VP2 genes (1755 nucleotides) were PCR amplified from the three viruses contained in the original faecal samples. Comparative analysis of the deduced VP2 proteins showed no obvious changes with respect to other type 2b CPVs retrieved from the GenBank database or analysed in a previous study (Decaro et al., 2009). Twelve of the 16 samples presenting type 2c CPVs that had been sequenced (Decaro et al., 2009) gave positive results by the in-house test. Analysis of the VP2 sequences from the positive samples showed the presence of mutations that were recently reported, including changes Ser to Ala at position 297 and Ile to Val at position 555. Two CPV-2c samples, 208/07 and 243/07-1, shared an additional mutation (Arg to Lys) at position 274, but they tested negative and positive, respectively, by the SNAP Parvo assay. The remaining three SNAP Parvo negative type 2c CPVs did not exhibit additional mutations with respect to the positive samples. The single CPV-2a sample whose VP2 sequence was available tested positive by the SNAP Parvo test. This strain contained the mutation Thr to Ala at residue 440, which has been recently reported in some CPV mutants worldwide, including CPV-2c strains circulating in the USA (Decaro et al., 2009).

Discussion and conclusions

In order to assess the ability of the SNAP Parvo test to detect the CPV antigenic variants, we have tested specimens representative of the three types, including 100 samples containing the recently detected CPV-2c. We selected samples of the different CPV types containing viral loads higher than 10^5 DNA copies/mg faeces to minimise the influence of the viral titre within the CPV sample population on the SNAP results. The detection rate of CPV-2c was not different from those of the other two CPV types, although a definitive comparison of the test sensitivity for detection of the different variants should be obtained by testing of samples with lower viral titres. At the same time, some important mutations detected more recently were proven not to affect the ability of the monoclonal antibody to recognise to the mutant strains. While the rapid test routinely detected samples containing viral loads higher than 10^5 DNA copies/mg faeces (>75%), we were surprised to find three British CPV-2b samples containing very high viral loads that tested negative by the in-house assay. The test positive results obtained with two viral isolates and the absence of any changes in the major capsid protein of the three viruses (including the strain that was not adapted to grow in vitro) may suggest the presence of high titres of interfering antibodies in the samples or, less likely, the extensive degradation of the capsid antigens related to the long-term storage. In conclusion, the SNAP Parvo test was proven to detect the new variant CPV-2c. However, considering the higher sensitivity of the molecular methods, the recommended approach for parvovirus diagnosis is to utilize the in-house assay first followed by submission of the faecal samples to the laboratory for the PCR-based assays in questionable cases.

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VALIDATION OF DEVELOPED AIV REAL-TIME RT-PCR ASSAYS THROUGH PARTICIPATION IN INTERLABORATORY COMPARISON TESTS

Maia ELIZALDE, Maria YUSTE, Marisa ARIAS, Jovita FERNÁNDEZ-PINERO

Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, Madrid, Spain

Keywords: Avian Influenza, real-time RT-PCR, validation

Introduction and Objectives

Avian Influenza (AI) is a highly contagious infection caused by viruses of the family *Orthomyxoviridae*, genus Influenzavirus A. Influenza A viruses are responsible for major disease problems in birds, as well as in mammals including humans. Different RT-PCR methods have been described for detection and characterization of avian influenza virus (AIV) isolates[1-6, 8-10]. RT-PCR test methodologies should be validated to the OIE standard to demonstrate the tests as being 'fit for purpose' for application in a field diagnostic setting[7]. Blind ring trials are periodically conducted to validate the PCR methods in use in the European laboratories[1]. Recently, two real-time RT-PCR methods for AIV detection were developed in our laboratory. One of them was designed within the nucleoprotein (NP) viral gene to cover a generic AIV detection[12]. The second one was focused to get a H7-subtype specific identification, including both American and Eurasian viral lineages[11]. In this study, these two AI real-time RT-PCR methods have been evaluated through participation in two interlaboratory comparison tests (ILCT).

Materials and methods

Two collections of blind samples coming from the AI Community Reference Laboratory (CRL), Veterinary Laboratory Agency (Weybridge, UK), were employed in two ILCTs for the PCR methods evaluation. The first panel included 32 RNA samples that were provided in RNA safe buffer. The second one included 10 lyophilised samples to be resuspended in 1ml of sterile distilled water. Total nucleic acid was robotically extracted from these ten samples using *BioSprint 15* workstation *(Qiagen)*. For the generic detection of AIV, the recommended EU M-gene real-time PCR[9,13] *(OneStep RT-PCR kit, Qiagen)* and the in-house developed NP-gene real-time PCR[12] *(QuantiTect Probe RT-PCR kit, Qiagen)* were performed. For H7-subtype specific detection, both recommended EU H7 Eurasian real-time PCR[13] *(OneStep RT-PCR kit, Qiagen)* and our developed H7 real-time PCR[11] *(QuantiTect Probe RT-PCR kit, Qiagen)* were carried out.

Results

All AIV positive samples belonging to different H-subtypes, included in the two coded collections, were properly detected by using the developed AI NP real-time assay, showing very similar or even lower Ct values than those obtained by performing the EU AI M real-time PCR procedure. When performed both recommended EU or novel developed H7 real-time PCR methods, specific detection of all H7 positive samples was obtained, while samples containing any other viral H-subtype gave none Ct value. The two blind panels incorporated also some samples containing Paramyxovirus type 1 (Newcastle disease virus) or type 2, and samples containing no virus, which remained negative when analysed by the two evaluated methods.

Discussion and conclusions

The recommended EU molecular diagnosis of AIV, using real-time PCR, includes a first sample screening by detection of viral M-gene, followed by subtyping of the positive samples[13]. In this work, an AIV generic real-time PCR method designed within NP-gene and an AIV H7 specific real-time PCR assays have been validated trough participation in two ILCTs. Both evaluated in-house real-time PCR methods have shown to be specific for its purpose. Dilution studies with H5 and H7 AIV representative isolates indicated that the two evaluated assays have a high level of analytical sensitivity. The completed studies prove that both generic NP-gene and H7 type-specific real-time PCR methods could be alternative useful tools for the detection and identification of AIV isolates.

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FIRST DETECTION AND PARTIAL CHARACTERIZATION OF KOI HERPESVIRUS (KHV) IN SLOVENIA

Aleksandra Grilc FAJFAR[1], Ivan TOPLAK[1], Peter HOSTNIK[1], Vlasta JENČIČ[2]

[1]*University of Ljubljana, Veterinary Faculty, Institute for Microbiology and Parasitology, Virology Unit, Gerbičeva 60, SI-1115 Ljubljana, Slovenia*
[2]*University of Ljubljana, Veterinary Faculty, Institute for Breeding and Health Care of Wild Animals Fishes and Bees, Gerbičeva 60, SI-1115 Ljubljana, Slovenia*

Keywords: koi herpes virus, molecular epidemiology, RT-PCR

Introduction and objectives

Koi carp herpesvirus (KHV) is the pathogen of koi and common carp (*Cyprinus carpio*) responsible for its mass mortality in the last decade. The disease was first reported in Israel in 1998 (1). In the same year the first outbreaks have been recognized also in the USA (4). Today KHV is spread in USA, Indonesia, China, Japan and in many European countries and is recognized as a global problem (5). The susceptible fish species for KHV are koi and common carp, but not the other related cyprinid species such as common goldfish. The morbidity of koi and common carp could be 100 % and the mortality between 25 and 90 % of the affected populations. The targeted tissues for KHV are gills, kidney, spleen, brain and liver (4). The most severe outbreak of the disease is when the water temperature is between 22 °C and 27 °C. The infection could be latent and an outbreak of the KHV is stress depended. The sequencing results of four complete genes (KHV helicase, KHV triplex, KHV DNA polymerase and KHV major capsid protein gene) showed that KHV is closely related to the carp pox herpesvirus (*Cyprinid herpesvirus* 1, CyHV-1) and *haematopoetic necrosis herpesvirus* of goldfish (*Cyprinid herpesvirus* 2, CyHV-2). KHV represents a third *cyprinid herpesvirus* (CyHV-3) in the family *Herpesviridae* (2). The high level of homology among KHV isolates indicates a rapid global spread of one prominent virus isolate from a single or limited source (3). The virus isolation on the cell cultures is a difficult and time consuming method that is also not always reliable, however the PCR method has been proven as an effective technique for the detection of the viral DNA.

Since 2006 the KHV has been established in many European countries, in Slovenia we have adopted specific PCR method for the laboratory diagnosis and introduced the surveillance program for this serious infection.

Materials and methods

Fish samples

During the years 2007 and 2008 we investigated carps from 36 ponds for KHV. Tissue samples from these 36 units without clinical symptoms have been collected and tested for KHV by PCR method. In summer 2008 increased mortality of common carp in the typical extensive polyculture carp pond in Maribor region and few days later from Ptuj region was recognized. In both cases the moribund and dead carps were dissected. From the carp showing pathological signs typical of KHV gills, spleen and kidney were taken for the laboratory confirmation of the KVH.

DNA extraction

Approximately 25 mg of the gill, spleen and kidney tissue samples were taken from individual fish. DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Germany), following the tissue extraction protocol. The DNA was stored in elution buffer at 4 ºC until tested by PCR.

PCR assay

Thymidine kinase (TK) has been shown to be essential for virulence in herpes viruses and we chose this gen for target sequence of the specific PCR primers. The PCR assay for the detection of KHV viral DNA in tissue samples was based on the protocol of Bercovier et al. (2) with few modifications. The specific primer pair of KHV-TKf (5'-GGGTTACCTGTACGAG-3') and KHV-TKr (5'-CACCCAGTAGATTATGC-3') was designed to amplify 409 bp of TK gen of KHV, but not CyHV-1 and CyHV-2. The amplification was performed with Platinum Taq® DNA Polymerase (Invitrogen) according to the manufacturer's instructions. The reaction was performed in a total volume of 50 µl as follow: 5 µl of DNA template, 32,1 µl of nuclease free water, 5 µl of 5 x PCR buffer, 4 µl of 10 mM dNTP mix, 10 pmol of each primer and 0,4 µl of Platinum Taq DNA enzyme. The thermal profile for PCR was: initial step of 95 ºC for 2 min, followed by 40 cycles of 95 ºC for 30 sec, 55 ºC for 30 sec, 72 ºC for 45 sec and final extension was at 72 ºC for 7 min. PCR products were visualized in 1,8 % agarose gel with ethidium bromide. The size of each PCR product was compared to the 100-bp DNA ladder and interpreted as positive or negative according to the expected size of DNA fragment.

In separate PCR tube, additional internal control of DNA isolation and PCR amplification was simultaneously run for each testing sample, with β-actin (forward primer: 5'-AGACATCAGGGTGTCATGGTTGGT-3'; reverse primer: 5'-CTCAAACATGATCTGTGTCAT-3') as target (expected PCR product of 259 bp). The thermal profile and other PCR reagents for this PCR were identical to the KHV PCR.

Sequencing of PCR products

Positive PCR products were used for direct sequencing, using the KHV-TKf and KHV-TKr primers. To determine the similarity of Slovenian isolates with other published KHV isolates, basic local alignment search tool (BLAST), similarity searches were performed against GenBank data.

Results

In screening testing, during 2007 and 2008, carps from 36 carp ponds had no signs of KHV and were negative by PCR. In August 2008 we had two clinical outbreaks of KHV in two carp ponds; one in Maribor region and one in Ptuj region. Carps had typical signs of KHV: appetite loss, erratic swimming, discoloration, necrosis of gill filaments. By fish farmer's data the mortality rate during both outbreaks was between 10 - 30 %. In both cases KHV was confirmed by PCR. Four PCR products of 409 bp were successfully sequenced and 389 nucleotide of each isolate was compared with sequences in GenBank. Piar-wise comparison of sequenced region revealed that four Slovenian KHV isolates were closely related (100 % identical). The obtained sequence of KHV showed also 100 homology in TK gene (the nucleotide genome position between 12 and 400) with four KHV sequences available in GenBank; cyprinid herpes virus 3 (accession No. AB458384), strain TUMST1 (accession No. AP008984), strain KHV-U (accession No. DQ657948) and strain KHV-I (accession No. DQ177346).

Discussion and conclusions

In August 2008 two outbreaks of KHV were detected in Slovenia for the first time. KHV was highly virulent and induced mortality among all sizes of common carp. The PCR has proven to be fast and effective method for first detection of viral DNA in a number of fish tissues during the acute outbreak and screening program. Sequencing analysis of four Slovenian KHV isolates revealed 100 % homology, what is confirmation of the cross-infection between both units and infection with the known KHV in Europe. With regard to the increasing threat of spreading of the virus to the other locations in Slovenia intensive disinfection of all ''material in use'' and other control measures were implemented on both infected locations according to the Council Directive 2006/88/EC. The Veterinary Administration Republic of Slovenia (VARS) declared the entire county as the compromised area for the KHV and the owners of carp ponds, fishermen and other people were asked to report about the eventual other infections and outbreak of the KHV. The sequencing results over the world suggested, that KHV had already spread extensively via pet-trade and due to the global threat for koi and common carp intensive measurement have to be implemented to prevent spreading of KHV.

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ENZOOTIC BOVINE LEUKOSIS: WHAT INDICATIONS ARE SUITABLE TO STANDARDIZE AND VALIDATE THE MILK ELISA TEST FOR INDIRECT DIAGNOSIS?

Francesco FELIZIANI, Francesco VITELLI, Francesco CESARINI, Antonio DE GIUSEPPE

Istituto Zooprofilattico Sperimentale Umbria e Marche - Perugia (Italy)

Keywords: quality insurance, retroviruses, surveillance plans

Introduction and objectives

Enzootic Bovine Leukosis (EBL) is a disease of cattle caused by the Bovine Leukaemia Virus (BLV). This agent belongs to the Retroviridae family that includes viruses that are able to determine neoplasias in mammals, birds and reptiles. Any animal that develops antibodies is considered infected. The methods most widely used are agar gel immunodiffusion (AGID) on serum and the enzyme-linked immunosorbent assay (ELISA) on serum or milk. These tests have formed the basis for successful eradication policies in many countries. Serum ELISA is more sensitive than other serologic tests and may also be used on milk. ELISA may be used for pooled serum samples and allows detection of antibodies in herds with a prevalence of <1%. The bulk tank milk ELISA is useful for identification of herds that are negative for BLV infection.

Since 1996 legislation in Italy implements the provisions of Council Directive 77/391/EEC and 97/12/EEC. These Directives require the operation of a monitoring and testing programme in order to obtain and/or maintain officially EBL-free status. In fact a compulsory Eradication Plan was implemented and some regions were declared officially free from the disease: in these situations the objective is to verify the acquired sanitary status, thus reducing the costs of surveillance. A possible solution would be to reduce the number of samples to be tested, mainly through a reduction in the sampling frequency. The sensitivity of the ELISA kits in use at the moment for the indirect identification of EBL would also allow the use of different matrixes, such as bulk milk, that could reveal the presence of positive animals at farm level.

Since our national facility is based on accredited laboratories, it is a requirement that the ELISA tests be validated and all quality assurance tests maintained. The ISO 17025 standard also requires a system for regular analyst training using on-site proficiency panel testing to ensure that the laboratory consistently produces reliable test results. The Istituto Zooprofilattico Sperimentale Umbria Marche is the National Reference Laboratory (NRL) for ruminants Retrovirus; the NRL has the responsibility to verify the performances of ELISA kits and particularly to define the sensibility of the tests. Therefore the evaluation of laboratory performance is another pressing need, underlined by European legislation and by The International Organisation for Standardization. To reach both goals contemporary, the NRL organize, annually, an inter-laboratory comparison to evaluate the performances of laboratories involved in the national eradication plan or in regional surveillance plans and to standardize the ELISA kits they use routinely.

Even if various European and extra-European countries such as Finland (L. Nuotio et al. 2003), the United Kingdom, Israel and Canada (Sargeant et al. 1997) have already used surveillance systems for EBL based on the examination of bulk milk, data concerning a suitable protocol to evaluate the efficacy and efficiency of this method is not available and outlining the Italian experience would be a contribution to the discussion on this topic.

Materials and methods

The main reference used to investigate the performances of Milk ELISA tests available to investigate the presence of BLV infection was the World Organisation for Animal Health (OIE) Terrestrial Manual (Chapter 2.4.11. - Enzootic bovine leukosis). In this manual it is also suggested the use of weak positive and negative OIE Standard Sera. Recently the OIE experts have announced the calibration of new, accredited OIE Standard serum, named 'E05', which has been validated against the former Standard serum E4 by different AGID and ELISAs. These sera can be used to establish ELISA sensitivity. The OIE suggest that Strong positive, weak positive, negative milk and diluent controls should be included in each assay. The strong positive control should be prepared by diluting the OIE positive Standard Serum (E05) 1/25 in negative milk. The weak positive control should be prepared by diluting, in negative milk, the OIE positive Standard Serum (E05) 25 times the number of individual milk samples in the pool under test. The milk used for diluting the Standard Serum controls should be unpasteurised, cream free and preserved. The sensitivity of pooled milk ELISAs must be evaluated using the OIE weak positive and negative Standard Sera. Assays should give a positive result on OIE standard serum E05 diluted in negative milk 250 times more than the number of individual milks in the pool (EU Directive 88/406). For individual milk samples the positive OIE Standard Serum E05 diluted 1/250 in negative milk must be positive. No indications are provided to investigate the specificity and the reproducibility of the test.

Since 2005, more than 70 Italian laboratories participated in the inter-laboratory trial to evaluate the sensitivity and specificity of their routinely employed tests for the indirect diagnosis of BLV.

The NRL distributed a panel of samples containing eleven negatives bulk milk samples, six positive samples obtained by the dilution of milk derived from a BLV infected dam in negative milk and six positive samples obtained by the dilution of Italian standard serum in negative milk. This serum was calibrated against the E05 serum.

Three different kits for milk ELISA were used by the laboratories involved in the inter-laboratory comparison.

An informative system was implemented to collect the data and a statistical analysis was performed on the basis of the correspondence to expected results.

Results

The Ring Trial involved respectively 26 laboratory in 2006, 27 in 2007 and 29 in 2008. The results are summarized in the table 1.

Table 1: percentage of results in accordance to expected ones

Sample categories		% of correct results
negatives samples		100
E5 Serum dilution in negative bulk milk	1/250	100
	1/500	100
	1/1000	96.55
	1/1500	96.55
	1/2000	96.55
	1/10000	93.10
Pos. Milk (milked from an infected dam) dilution in negative bulk milk	1/10	96.55
	1/20	96.55
	1/40	93.10
	1/80	85.34
	1/160	68.10
	1/320	65.00

Discussion and conclusions

Milk ELISA sensitivity was found to be in accordance with international standards, but specially "home made" kits showed a low level of analytical sensitivity, even though in the ELISA Kits tested the values of specificity, repeatability and reproducibility were considered as acceptable. It seems that reference samples suggested by OIE experts are useful only to standardize the ELISA test but to investigate real values of sensitivity it is necessary to include other samples as shown in through Italian experience.

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DETECTION OF NEUROPATHOGENIC STRAINS OF EQUINE HERPESVIRUS 1 (EHV-1) ASSOCIATED WITH ABORTION IN GERMANY

Ann-Kathrin FRITSCHE, Kerstin BORCHERS

Institute of Virology, FU Berlin, Faculty of Veterinary Medicine, Philippstr. 13, 10115 Berlin, Germany

Keywords: EHV-1, abortion, neuropathogenic marker, ORF30, PCR

Introduction and Objectives
A single nucleotide polymorphism within EHV-1 gene ORF 30, which is coding for viral DNA polymerase, allows differentiation of the neuropathogenic [G_{2254}] from non-neuropathogenic biovar [A_{2254}] (Nugent et al., 2006; Goodman et al., 2007). The aim of the study was to investigate the distribution of the neuropathogenic marker in EHV-1 isolates from abortion cases diagnosed at the institute over the last three years.

Materials and Methods
30 EHV-1 isolates obtained between 2006 and 2009 from nine different studs with abortions were isolated from different tissues such as fetal lung, liver and spleen or from nasal swabs of mares. To determine the nucleotide sequence at the polymorphic site in gene ORF30, a gene specific PCR and subsequent SalI restriction enzyme digest were used as described by Allen (2006).

Results
Among 30 abortigenic EHV-1 strains 93.3% (28/30) were of non-neuropathogenic genotype but 6.6% (2/30) harbored the neuropathogenic marker. These strains were detected in nasal swabs of two pregnant mares with neurological symptoms and after abortion in fetal tissues.

Discussion and Conclusions
The ORF 30 PCR and subsequent SalI digest represent a reliable tool to differentiate non-neuropathogenic from neuropathogenic strains. Unexpectedly, 6.6% of the isolates from abortions were positive for the neuropathogenic allele (D752). Such exceptions to the rule should be analysed more extensively, and factors such as double infections or genetic background of the horse should be taken into further account.

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DETECTION OF BOVINE VIRAL DIARRHOEA VIRUS (BVDV) IN BOVINE ABORTIONS

David A. GRAHAM[1], Norman BEGGS[2], Karen MAWHINNEY[1], Maria P. GUELBENZU[1], Vi CALVERT[1], Ben CUNNINGHAM[1], Lynsey ROWAN-LAYBERRY[1], Ian MCLAREN[1]

[1]Agri-Food and Biosciences Institute, Veterinary Sciences Division, Belfast;
[2]Agri-Food and Biosciences Institute, Veterinary Sciences Division, Omagh

Keywords: bovine abortion, bovine viral diarrhoea virus

Despite the well recognised capacity of bovine viral diarrhoea virus (BVDV) to cause abortion in cattle, infection in foetal material can be difficult to diagnose.

To improve the diagnostic rate for BVDV infection in aborted foetuses, a panel of tests were applied to samples from 140 foetuses submitted for *post mortem* examination: immunofluorescent antibody testing [IFAT] on spleen ileum and lung; virus isolation on pooled spleen, ileum and lung; BVDV antigen ELISA on foetal fluid and ear notch; serology ELISA and real time RT-PCR on foetal fluid. Positive results from one or more tests were obtained from a total of 25 (17.9%) foetuses. IFAT and virus isolation gave positive results in only 1/134 (0.8%) and 3/134 (2.2%) and foetuses respectively. In contrast, 20/140 (14.3%) foetal fluids and 16/129 (12.4%) ear notch samples were positive by antigen ELISA. Real time RT-PCR was positive in 18/138 (13%) foetal fluids. Virus-specific antibodies were detected in 2/140 (1.4%) samples.

In only six of the twenty-five foetuses testing positive for BVDV were possible alternate causes of abortion detected.

The results show that use of the traditional diagnostic methods of virus isolation and IFAT significantly underestimates the presence of BVDV in abortions when compared to antigen ELISA or real time RT-PCR. These findings emphasise the prevalence of BVDV infection in foetuses and the need for systematic control at the herd level.

VALIDATION OF A COMPETITIVE ELISA FOR SERODIAGNOSIS OF PRRS BASED ON RECOMBINANT N-PROTEIN AND MONOCLONAL ANTIBODY

Santina GRAZIOLI, Giulia PEZZONI, Paolo CORDIOLI, Emiliana BROCCHI

Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna, Brescia Italy

Keywords: antibodies, ELISA, monoclonal antibodies, PRRS, recombinant N-protein

Introduction and objectives

Porcine Reproductive and Respiratory Syndrome (PRRS) is one of the most economically important disease of swine, caused by an arterivirus. The viral nucleocapsid protein N is a strong inducer of antibodies and thus represents a suited antigen for serological assays. The objective of this study was the development of a specific and sensitive competitive ELISA based on a recombinant N protein expressed by E.coli and one specific monoclonal antibody (MAb).

Materials and methods

Expression of N protein. ORF7 gene (386pb) corresponding to N protein from the Italian field isolate IT2156/92 of PRRSV was reverse transcribed, amplified using specific primers and ligated into pQE30 vector (Qiagen). The ligated plasmid was used to transform BL21 pREP4 competent cells. Individual colonies were selected and tested for the presence of the desired insert by sequencing. The selected colony was grown at 37°C with vigorous shaking in LB broth supplemented with ampicillin (100ug/ml) and kanamicin (25ug/ml) until the midlogarithmic phase. Isopropylthiogalactoside (1mM) was added and the culture was incubated for additional 3 hours to induce the expression of the recombinant protein. Cells were harvested and lysed by sonication in a non denaturing buffer.

Anti N protein MAbs. A panel of 5 anti-N MAbs (2B2,3B1,1D8,4D5,4D8) raised against the Italian strain PRRSV 2156/92 (Cordioli et al.) was selected for antigen characterization. MAbs were peroxidase-coniugated.

Characterization of N protein. The reactivity of the recombinant protein with MAbs was evaluated in different assay systems: by Western blotting against SDS-PAGE separated cell lysates, electrophoretically transferred onto PVDF membranes, by direct ELISA against antigen coated to the ELISA solid phase and by competitive ELISA to evaluate the reciprocal competition between MAbs or between positive sera and MAbs. In the latter assay, each MAb or immune sera was individually incubated with coated N antigen simultaneously with a peroxidase-conjugated MAb.

Competitive ELISA for antibody detection. A crude bacterial cells lysate was adsorbed onto NUNC maxisorp microplates at saturating concentration (5 µg/ml). Diluted sera and the peroxidase-conjugated MAb 4D5, pre-calibrated in order to give a fixed optical density of 1.5 units, were sequentially incubated. After washing, the chromogenic reaction was developed and results were expressed as percentage of competition of the MAb binding to the N protein caused by sera.

Sera. The following sera were examined:

- 1031 field swine sera from herds in northern Italy;
- a panel of 246 experimental sera, sequentially collected from 18 pigs: 12 pigs were vaccinated with a commercial attenuated vaccine, 6 pigs were not vaccinated; subsequently all the animals were challenged in two occasions with genotipically distinguishable viruses.

Commercial kit. The commercial kit HerdChek PRRS 2XR antiboby ELISA (IDEXX, Westbrook, Maine) was used as a comparative test. The kit has gained wide acceptance in diagnostic community and it is the most used serological test for PRRS diagnosis. The principle is that of an indirect ELISA based on two recombinant N proteins obtained from European and American PRRS strains as antigen and on anti-swine immunoglobulins as reaction detector.

Results

N protein characterization. Highest yields (40 mg/L of culture) of the recombinant N protein were obtained after three hours of bacterial culture induction; given its easy solubility the protein could be recovered in a native buffer, preserving the proper folding. The recombinant antigen was recognised by all the five MAbs in both direct ELISA and Western blotting (fig.1). Results of reciprocal competition assays suggested that these MAbs identify four distinguishable epitopes; three of them, target of 4D8, 4D5 and 3B1/2B2, are independent each other, whilst epitope target of MAb 1D8 seems to have structural relations with both 4D5 and 3B1/2B2 epitopes. In prototypes competitive ELISAs, PRRS-positive sera efficiently inhibited binding of MAbs 4D5 and 3B1/2B2 to the N protein, suggesting that they recognise highly immunogenic domains and then are good candidates for setting of a competitive test. Among them MAb 4D5 was finally selected for the competitive ELISA, as the involved epitope is common to both European and American PRRS strains.

Competitive ELISA performance. In preliminary feasibility studies, serial dilutions (from 1/5) of a sub-set of sera were examined; results led to fix an optimal screening dilution of 1/15 and a provisional cut-off of 50% competition. Testing of 1031 field sera, classified as positive or negative according to their reactivity in IDEXX HerdChek kit, confirmed that 50% competition is the optimal cut-off (fig.2): concordance between the competitive ELISA and IDEXX HerdChek was 93%. Forty-one out of 643 IDEXX-positive sera, most of them (n°29, 70.7%) borderline, scored negative in competitive ELISA; differently, 32 out of 388 IDEXX-negative sera reacted positive in competitive ELISA. Interestingly, 15 of these were from the same herd with a high seroprevalence.

The two tests were compared to evaluate kinetic of the immune response in experimentally vaccinated and infected pigs: concordance was good but the seroconversion was earlier detected by the competitive ELISA: 14 days post-vaccination this assay identified 10 positive animals out 12 vaccinated, whilst IDEXX HerdChek detected only 5 pigs.

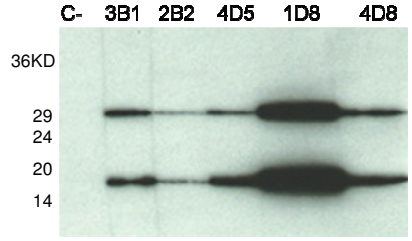

Fig.1: Reactivity of five anti-N protein MAbs in Western-blotting; the lower signal corresponds to N-protein (monomer), the upper one corresponds to protein dimers. C-: non-specific control MAb

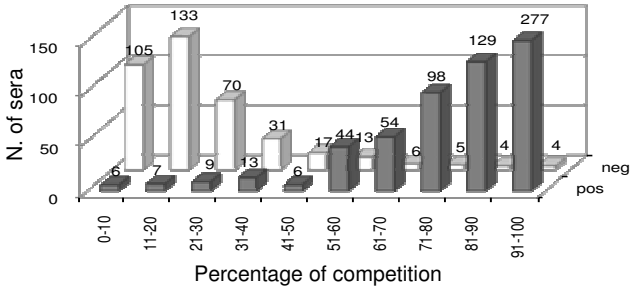

Fig. 2: Distribution of percentages of competition generated in competitive ELISA by 1031 swine sera, classified as positive and negative according to their reactivity in IDEXX HerdChek kit

Discussion and conclusions

Despite based on a different principle, the simple competitive ELISA developed for the detection of anti-PRRS antibody proved diagnostic performance similar to that of the widely used HerdCheK IDEXX ELISA, showing 93% concordance. The new assay detects immunoglobulins regardless of the antibody class and reveals seroconversion earlier than the commercial test, that is likely less sensitive to the IgM antibody class. Finally, it benefits from the use of reproducible reagents, such as the recombinant antigen and the MAb, enabling improvement of standardisation.

Acknowledgements

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EPIDEMIOLOGICAL SURVEY OF PATHOGENS ASSOCIATED WITH PORCINE RESPIRATORY DISEASES IN PIGS FROM NORTH-WESTERN GERMANY FROM 2007 TO 2009

Beatrice GRUMMER[1], Ludwig HAAS[1], Elisabeth Grosse BEILAGE[2], Heiko NATHUES[2], Irene GREISER-WILKE[1]

[1]Institute of Virology, University of Veterinary Medicine Hannover, Germany;
[2]Field Station for Epidemiology, University of Veterinary Medicine Hannover, Bakum, Germany

Keywords: epidemiological survey, infectious agents, multiplex PCR, porcine respiratory disease

Introduction and objectives

Respiratory diseases of swine are of major economic impact to the pig industry. They are often the result of a combination of primary with opportunistic infectious agents. In addition, adverse environmental and management conditions play an important role in the multifactorial nature of respiratory diseases in pigs. Modern diagnostic approaches must therefore take into account the presence of several pathogenic and opportunistic microbial agents. Since two years a random-primed, two-tube reverse transcriptase multiplex RT-PCR targeting six viral agents and two mycoplasma species and is in use in the routine diagnostic of the Institute of Virology (Harder, 2004).

To determine the relative frequency and the frequency of co-infections of the different agents that are currently associated with respiratory diseases in pigs, we evaluated retrospective data from 2007 to 2009.

Materials and methods

Diagnostic materials were nasal swabs, broncho-alveolar lavage fluids (BAL), and lung/lymph node tissues of porcine origin submitted for routine examination. Affected herds and animals were located in north-western Germany (Lower-Saxony and Northrhine-Westphalia), an area.

The RT-PCR assay can detect *porcine circovirus 2* (PCV-2), *porcine reproductive and respiratory syndrome virus* (discerning European and North-American genotypes) (PRRSV-EU and US), *porcine respiratory coronavirus* (PRCV), *swine Influenza virus* (SIV), *porcine cytomegalovirus* (PCMV) and the mycoplasma species *hyopneumoniae* and *hyorhinis*. Transcripts of the porcine single copy gene *ppk98* were used as an internal extraction and amplification control. The multiplex PCR is based on RNA isolation and depends on reverse transcription and PCR in two-tube reaction. The genomes of mycoplasms, PCMV, and PCV-2 are DNA whereas all other viral targets have an RNA genome. Therefore, the detection of mycoplasms, PCMV, and PCV-2 is largely dependent on their replication, i. e. the presence of mRNA, which is considered to be more relevant for the involvement in disease (Yu et al., 2005)

For the extraction of total RNA the RNeasy Mini kit was used as recommended by the manufacturer (Qiagen). Reverse transcription of the RNA was achieved by use of Superscript II (Invitrogen) and random hexamer priming (Liermann et al., 1998). PCR was done as follows: 95 °C for 15 min, 35 x [94 °C for 30 sec, 57 °C for 90 sec, 72 °C for 60 sec], 1 x [72 °C for 10 min]. Amplificates were size fractionated in 3 % agarose gels.

Results

More than 1500 clinical samples obtained from pigs suffering from respiratory signs were examined by the multiplex assay. In one third of the samples none of the pathogens were detected, whereas two thirds were positive for at least one of the tested agents. Most frequently, PRRSV-EU (31 %), PCV-2 (35 %), and M. *hyorhinis* (35 %) were found, whereas PRCV and PCMV were rarely found.

Half of the infected animals harbored one pathogen, one third was infected with two pathogens, about 10 % were co-infected with three pathogens and a minority was positively tested for four or even five of the agents.

Discussion and conclusions

From the results above it is confirmed that much of the importance of respiratory diseases in pigs is due to the interactions between pathogens. Previous studies demonstrated that the etiology of respiratory diseases varies between and within production systems and over time within the same system. Very often one or two viruses, M. *hyopneumoniae*, and several opportunistic bacteria worked in combination to induce losses associated with respiratory disease. In addition to the pathogens long known to induce respiratory disease, several emerging and changing pathogens play an important role in the development of porcine respiratory disease complex. The emergence of PRRSV in the late 1980s resulted in significant changes in the health status of the worldwide swine population. In addition to PRRSV, several other respiratory pathogens have emerged, including PCV2, PRCV, and new strains of SIV (Brockmeier et al., 2002). These observations underscore the importance of conducting a complete diagnostic investigation, even if one respiratory pathogen has been identified.

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COMPARISON OF FOUR REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION ASSAYS FOR THE DETECTION OF BOVINE VIRAL DIARRHOEA VIRUS NUCLEIC ACID

Maria P. GUELBENZU, David A. GRAHAM

Agri-Food and Biosciences Institute, Veterinary Sciences Division, Belfast

Keywords: bovine viral diarrhoea virus, real time RT-PCR

Introduction and objectives
Four commercially available TaqMan fluorogenic probe-based one-step real-time reverse transcription polymerase chain reaction (real-time RT-PCR) assays (A, B, C, D) were compared for the detection of Bovine Viral Diarrhoea Virus (BVDV) nucleic acid in tissue culture supernatant, serum and milk pellet.

Materials and methods
Three culture derived pestivirus strains (BVDV Type 1 [NADL], BVDV Type 2 [890] and Border Disease Virus [S137]), three BVDV antigen ELISA positive field sera and one BVDV virus positive milk pellet were selected. Viral nucleic acid was extracted, diluted, aliquoted and stored at -20°C. The real time RT-PCR Kits were used according to manufactures' instructions.

Results
In general, all the kits were able to detect pestivirus RNA in all samples. Differences between kits were noted in terms of limit of detection/analytical sensitivity. Overall kit B was more sensitive for BVDV Type 1 and 2 but performed last well with BDV. All kits had a dynamic range of 10^4. Linearity ranged from 0.908 for Kit D to 0.998 for kit B for BVDV Type 1. Efficiency of amplification based on BVDV 1 and 2 ranged from 100.072% for kit B to 151.05% for kit D.

Discussion and conclusions
Kit B was selected for further use within the laboratory.

DETECTION OF ASFV USING LATE-PCR AND ROTARY DESIGN AMPLIFICATION SYSTEM

Mikhayil HAKHVERDYAN[1], Bonnie RONISH[2], Carmina GALLARDO[3], Jovita FERNÁNDEZ-PINERO[3], Karl STÅHL[1], Neil LEBLANC[1], Lawrence WANGH[2], Sándor BELÁK[1]

[1]National Veterinary Institute (SVA), Department of Virology, Immunology and Parasitology, Uppsala, Sweden;
[2]Brandeis University, Department of Biology Waltham, MA, USA;
[3]CISA-INIA, Ctra Algete el Casar s/n, Valdeolmos, Madrid, Spain.

Keywords: ASFV, endpoint detection, LATE-PCR, Rotor-Gene

Introduction and objectives

African swine fever virus (ASFV) is a causative agent that infects domestic pigs and results in highly contagious disease with high mortality rates associated with economic losses. It is the only known DNA arbovirus that has been classified as the sole member of family *Asfarviridae (African swine fever and related viruses),* genus *Asfivirus* (Tulman et al., 2009). According to the World Organisation for Animal Health (OIE) classification, African swine fever (ASF) is regarded as a notifiable disease. Endemic in Africa until 1957, the disease spread rapidly to the north, with ease crossing borders of many European countries, and recently, Georgia, Armenia, Azerbaijan and Russia (2007-2008) (Rowlands et al., 2008). ASFV crossed the Atlantic Ocean during the late 1970s causing outbreaks in the Caribbean and Brazil.

The first PCR assay that applied for the detection of ASFV was developed in 1992 (Steiger et al., 1992). From this time several conventional, nested and real-time PCR assays were developed (Agüero et al., 2003; King et al., 2003; Zsak et al., 2005; Basto et al., 2006; McKillen et al., 2007; Wilkinson; 2008; Giammarioli et al., 2008). The objective of this study was development and evaluation of the Linear-After-The-Exponential (LATE)-PCR for detection of ASFV based on a new principle of primer design, which in its turn, gives several advantages over eight PCR assays developed earlier between 1992-2008.

Materials and methods

Fourteen ASFV strains from Africa, Italy, Spain and one from Haiti were obtained from CISA-INA (Valdeolmos, Madrid, Spain) in the frame of ASFV EU-project. DNA was extracted from infected cells using a High Pure PCR template preparation kit (Roche Molecular Biochemicals) and supplied to the SVA. ASFV specific primers and probe targeting the p72-gene of the GenBank (NCBI) published sequence were developed in the Wangh Laboratory at Brandeis University (MA, USA) using Visual-OMP (Oligonucleotide Modeling Platform) package from DNA Software, Inc. (Ann Arbor, MI, USA). An assay internal control consisting of 85 bp synthetic DNA target with corresponding primers and probe was designed, as well. The sensitivity of the LATE-PCR was determined using a 101 bp synthetic DNA template. DNA carrier (10 µg/ml salmon sperm DNA, Ambion, Austin, TX, USA) was used to prepare 10-fold dilution series in TE-buffer to assure a constant amount of nucleic acids in the diluted samples. A Rotor-Gene 3000 PCR instrument (Qiagen; formerly Corbett Research, Australia) was used for the assay evaluation and optimisation. Smiths Detection (UK) has an exclusive license agreement with Brandeis University for LATE-PCR and related technologies.

Results

Designed assay was optimised by means of reaction conditions and thermodynamic profile until it showed the highest fluorescence signal, specific melting peaks and maximum differentiation between positive and negative samples. In contrast to the traditional real-time PCR, the ASFV LATE-PCR is based on the endpoint detection, which means that 50 cycles of amplification are followed with one cycle at 70°C, 50°C and 40°C, each, where fluorescence data are collected. Melting profile analysis directly after the PCR confirms positive or negative amplification. All 14 tested ASFV strains were detected, which was revealed by increased fluorescence signal for ASFV targets, followed by melting curve analysis directly after PCR, with determination of probe melting point. Probe melting also confirmed specific hybridisation of the ASFV strains. The analytical sensitivity of the assay was one copy of viral genome equivalent which was determined after 10-fold dilution series, in triplicates. ASFV LATE-PCR was also tested in duplex format, together with the internal control and PrimeSafe™ reagent. ASFV target and internal control, both were successfully amplified, while PrimeSafe™ drastically reduced all forms of mispriming and resulted in the specific amplification product only.

Discussion and conclusions

LATE-PCR is an advanced method of asymmetric PCR that uses a new approach for the primer design and the endpoint amplification technique (Sanchez et al., 2004; Pierce et al., 2005). Linear amplification of one strand occurs after the limiting primer is depleted. In the absence of a complementary strand the single-stranded product can be detected at endpoint over a wide range of temperatures (down to 35°C) using low temperature probes which do not interfere with exponential amplification during the first phase of the reaction (Pierce, Wangh, 2007). The resulting fluorescence signals are brighter. LATE-PCR assays can readily be multiplexed by addition of PrimeSafe™ and the single-stranded amplicons can be sequenced using a simple Dilute-'N'-Go procedure (Rice et al., 2007). The enhanced features of LATE-PCR compared to conventional real-time PCR result in more robust detection and analysis of ASFV. The assay is sensitive, since it can detect one copy of viral genome equivalent. Thus the assay may provide an effective tool to trace ASFV in early stages of infection during the outbreaks. The assay can easily be adapted to the portable PCR machine using endpoint detection features that may bring more simplicity and flexibility in ASFV LATE-PCR performance. Usage of low melting temperature probes and assay effective performance, for example at 35°C, could allow detection of ASFV strains with mutations in the probe region. In the future, this technique is going to be adapted to the BioSeeqVet portable PCR instrument (Smiths Detection, UK) for the direct detection of ASFV in the field conditions.

In conclusion, the high sensitivity and specificity of the ASFV LATE-PCR assay, together with all benefits of LATE-PCR technology mentioned above, will improve the early and rapid detection of a wide range of ASFV strains, allowing reduced turnaround time and use of high-throughput, automated technology.

Acknowledgements

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ELISA FOR DETECTION OF AVIAN INFLUENZA H5 AND H7 ANTIBODIES

Trine H. JENSEN[1], Gitte AJJOURI[1], Kurt HANDBJERG[1], Marek SLOMKA[2], Vivien COWARD[2], Martine CHERBONNEL[3], Poul H. JØRGENSEN[1]

[1] National Veterinary Institute, Technical University of Denmark Department of Poultry, Fish and Fur Animals, Aarhus, Denmark;
[2] Veterinary Laboratories Agency, Departmen of Virology, Surrey, UK;
[3]National Reference Laboratory for avian influenza and Newcastle disease, poultry and rabbit virology immunology and Parasitology, AFSSA, Research Laboratory on Poultry, Swine and Fish Unit, Ploufragan, France

Keywords: antibody, avian influenza, H5, H7, inhibition ELISA

Introduction and objectives
Avian influenza is an emerging global challenge regarding the potential for pandemics with severe impact on the avian health and economy[1].Of special concern is the avian influenza virus (AIV) subtype H5 and H7, which has the potentials to become highly pathogenic avian influenza (HPAI)[2]. The zoonotic potential of H5 and H7 infections further emphasise the need for sensitive and effective diagnostic methods and surveillances. National serological surveillance programmes often rely on the haemagglutination inhibition (HI) test. However, for screening of high numbers of samples the ELISA techniques are superior in throughput and speed. Antibody ELISA techniques for detection of antibodies against the AIV NP[5], H5[3], H7[4] has been described. Nevertheless, there is an increasing demand for these techniques to be validated, and we have developed H5 and H7 monoclonal antibodies for use in ELISA and immunohistochemistry. Here we describe an inhibition ELISA employing the H5 and H7 monoclonal antibodies which proved to be as sensitive as the HI test.

Materials and methods
H5 and H7 monoclonal antibodies (mAbs) were developed by immunisation of mice with inactivated A/ostrich/DK/72429/96 (H5N2 LP) and a DNA plasmid encoding the H gene of A/Chicken/Italy/1067/99 (H7N1 LP), respectively. The mAbs were evaluated against 47 different H5, H7 and other AI subtypes by ELISA and immunohistochemistry. The H5 and H7 mAbs were used as secondary antibodies in the development of an inhibition ELISA. In brief, ELISA plates were coated with inactivated A/mallard/DK/64650/2003 (H5N7) and A/African starling/Eng/983/79 (H7N1).

The plates were incubated with experimentally derived chicken anti-serum against AIV subtype H1N2, H5N2, H5N7, H7N1, H7N7, H9N9, H10N4 and H16N3 (table 1) followed by mAbs. The reaction was made visible by horse-radish-peroxidase conjugated polyclonal rabbit anti-mouse IgG and OPD.

The HI test was performed as described in the EU Diagnostic Manual (Council Directive 1992/40/EC).

Results
This ELISA was specific as only sera against AIV subtype H5 and H7, respectively were recognised. The H7 ELISA was more sensitive than HI as serum with low titers was detected by ELISA and not by HI.

Discussion and conclusions
The inhibition ELISA described here was as efficient as the HI test and further has the advantages of being fast in performance and having a high throughput. Additionally, the presented H5 and H7 monoclonal antibodies can also successfully be used for immunohistochemistry. Future aims are to validate this ELISA against other avian sera and potentially also against other commercial or published ELISA techniques [3, 4, 5].

Acknowledgements
The technical assistance of Lisbeth Nielsen and Hanne Røigaard is highly appreciated. This work was founded by the EU project FLUTEST and AVIFLU.

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Table 1. Chickens immunised with avian influenza for production of chicken anti-serum

Avian influenza isolates used for immunisations	Origin of isolate	Chicken	Number of chickens
A/Swine/DK/13608/04 (H1N2)	VET	SPF	15
A/ostrich/DK/72429/1996 (H5N2)	VET	SPF	15
A/ostrich/DK/72429/1996 (H5N2)	VET	Broiler	15
A/chicken/Bel/150/99 (H5N2)	VLA	SPF	15
A/mallard/DK/64650/2003 (H5N7)	VET	SPF	15
A/African starling/Eng/983/79 (H7N1)	VLA	SPF	17
A/African starling/Eng/983/79 (H7N1)	VLA	Broiler	10
A/Turkey/Eire/95 (H7N7)	VLA*	SPF	15
A/Knot/Eng/02 (H9N9)	VLA*	SPF	14
A/Turkey/Eng/384/79 (H10N4)	VLA	SPF	16
A/Gull/DK7468110/2002 (H16N3)	VET	SPF	12

VET- National Veterinary Institute, Denmark
VLA – Veterinary Laboratories Agency, United Kingdom
SPF Specific pathogen free
* received inactivated and used directly

VHS OUTBREAKS IN FINLAND 2000-2008

Sirkka-Liisa KORPENFELT, Tiina NOKIREKI, Ilona LAAMANEN, Tuija GADD

Evira, Research Department, Veterinary Virology, Helsinki, Finland

Keywords: diagnostics, RT-PCR, VHS virus, virus characterisation

Introduction and Objectives

Viral haemorrhagic septicaemia (VHS) is a severe transmissible viral fish disease which affects mostly farmed rainbow trout. VHS virus belongs to the genus *Novirhabdovirus*, within the family *Rhabdoviridae*. In Finland measures to prevent the spread of the disease begin immediately when an infected fish farm has been found. Finland started the surveillance and control program in the year 1995.

Materials and methods

The total number of fish farms in operation in coastal and inland waters in Finland is 501. 201 of them are food fish farms, 108 are fry farms and 235 farms are natural food pond producers. Number of fish farms in coastal waters is 151 and in inland waters 350.

VHSV is examined in pooled homogenized tissue samples of a maximum of 10 fish. The primary diagnostic method is cell cultivation in BF-2 and EPC-cell lines. The cultivation time is two weeks. Cells are passaged once. If viral cytopathic effect (CPE) develops, the identification of the virus is made by ELISA kit according to the manufacturer´s instructions (Test-Line). Other confirmation method in use is RT-PCR.

Results

After the first VHS outbreak in the year 2000, the disease has been found every year in Finland. The main restriction area is the province of Åland, a group of islands, situated between Finland and Sweden. Second area where infection was found at the same year was Pyhtää on S-E coast, 70 km from the Russian border. Eradication in Pyhtää was successful and restriction area was cancelled in June 2008. The third location where the eradication measures were submitted in 2003 was on the coast of Gulf of Bothnia in Uusikaupunki area which is located on the west coast of Finland. This case had a confirmed contact with Åland. The area still remains as a restriction area; VHS was found there again in 2008.

The number of infected farms in recent years (new /old farms): 2005: 2/7, 2006:0/10, 2007: 0/2, 2008: 1/3. VHSV strains isolated from rainbow trout in Finland belongs to the genotype Id. Pair-wise comparisons of the G and NV gene regions of Finnish VHSV isolates collected between 2000-2004 revealed that all isolates were closely related, with 99.3 to 100% nucleotide identity, which suggests the same origin of infection. Luckily the fish in inland waters has remained free from the disease.

Discussion and conclusions

One hypothesis is that wild fish has been the origin of the first VHS outbreak in Finland. More research is still required to confirm the origin of VHS outbreaks. VHS outbreaks started from Åland. The worst year has been the year 2002 with 12 new VHS-positive fish farms. VHSV is found from 2-14 farms annually. In Åland Islands VHS outbreaks are regular and we don´t believe this restriction area to be cancelled in near future. Finland is considered VHS free apart coastal zones.

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LATERAL FLOW TECHNOLOGY – FOR A QUICK ON-SITE DIFFERENTIATION OF FMDV AND OTHER VESICULAR DISEASES.

Therese KRISTERSSON[1], Ann NORDENGRAHN[1], Malik MERZA[1]

[1]Svanova Biotech AB, Uppsala Science Park, Uppsala, Sweden

Keywords: FMDV, lateral flow technology, SVDV, vesicular diseases, VSV

Introduction and objectives

Consistent with the guidelines of the new policy oriented research is the development of rapid, simple and robust tests that could be used as first line diagnostics. Pen-side tests represent tools that satisfy these requirements: their application could be useful as a first line diagnosis for veterinarians in slaughter houses, in farms and in simply equipped regional laboratories.

The on site assays are mainly based on antigen or antibody recognition; consequently they use antibody-based detection systems.

The aim of this study was to develop Pen-side tests to quickly detect positive cases of FMDV and also to exclude two similar vesicular diseases, namely VSV and SVDV.

Materials and methods

- For each assay the FMDV, VSV Ind, VSV NJ or SVDV specific mAb was bound to a latex or gold carrier as well as immobilised on a nitrocellulose membrane. The mAb´s were kindly supplied by Dr. E Brocchi, IZLER, Brescia, Italy and by DR. N Ferris, IAH, Pirbright, UK.

- If virus is present in the sample it will bind to the carrier and form an immune complex.

- The complex then migrates by capillary action along the membrane until it reaches the immobilised antibody on the membrane.

- The complex will bind, resulting in a coloured line.

- Inactivated viral cell cultures from FMDV (sero type O, C, A, Asia 1, Sat 1, Sat 2, Sat 3), VSV Indiana1, VSV New Jersey and SVDV as well as normal non infected cell cultures were analysed in order to evaluate the assays sensitivity and specificity. The samples were kindly supplied by N Ferris, IAH, Pirbright, UK.

Results

Each assay showed 100% specificity with the homolog viral antigen while no reaction could be seen using samples from the other two viruses.
The reaction was read within 10 minutes.

Discussion and conclusions

We have successfully used the lateral flow technology in order to develop test system for FMDV, VSV and SVDV that could easily distinguish between the three viruses, all causing to some degree similar clinical signs. By using these Pen-side tests a yes/no answer could be obtained within 10 minutes giving the responsible Veterinarian the ability to quickly take the right precautionary measures in the matter.

POTENTIAL DIVA DIAGNOSTICS FOR AVIAN INFLUENZA: A COMPARISON AND VALIDATION STUDY

Katrin KÜHN, Martin BEER

Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, 17493 Greifswald-Insel Riems

Keywords: avian influenza virus, DIVA, ELISA

Introduction and objectives

In poultry the use of vaccination to control avian influenza (AI) is being considered as an appropriate tool and an alternative control strategy to stamping out. But conventional vaccines can affect serologic surveillance, and in addition have negative impact on international trade. For both surveillance purposes and trade, it is important to differentiate infected from vaccinated animals (DIVA) using DIVA (marker)-vaccines. Because marker vaccine technology is only as good as its companion diagnostic test, it is of utmost importance to have validated, reliable DIVA test systems available.

Materials and methods

Presently, numerous diagnostic tests are approved for detecting antibodies specific for avian influenza virus. Among them the indirect immunofluorescence assay (iIFA) as well as competitive or indirect enzyme-linked immunosorbent assays (ELISA) could have the capability of DIVA test systems. Therefore, we analysed three different ELISA systems, detecting antibodies against non-structural protein 1 (NS1) or neuraminidase 1 (N1) or nucleocapsid protein (NP) for their suitability as a DIVA test system. While NP assays can only be used in combination with recombinant vaccines (vector- or subunit-vaccines), N1 or NS1 ELISAs have a marker test potential also in combination with conventional inactivated avian influenza vaccines.

In order to validate these potential DIVA tests, a panel of 797 sera from chicken, geese, ducks, and turkeys was tested. The serum panel covered a wide range of different hemagglutinin and neuraminidase subtypes as well as viruses important for differential diagnostics (e. g. NDV). In addition, an iIFA test, based on recombinant baculovirus expressing neuraminidase 1 (N1) was established and compared with three commercially available ELISA systems.

Results

For the comparison of all assays, especially the parameters sensitivity and specificity were calculated. As a result, a clearly different suitability of these tests has been observed. While the ELISA tests which allow a species independent application like the competitive NP or N1 antibody ELISAs displayed good to acceptable sensitivity and specificity, the indirect assay for the detection of NS1 antibodies was restricted to the application of chicken sera, and showed a comparatively reduced sensitivity of less than 30 %. In addition, the iFA test was very useful as a control assay for the N1-ELISA.

The different sensitivities (from 29 % to 100 %) and specificities (from 77% to 98 %) of the investigated test systems will be presented and discussed. Furthermore, a recommendation for the use of the different marker tests in control strategies will be given.

Discussion and conclusions

As a conclusion, some of the investigated assays have a potential as candidate DIVA tests, however, with different capabilities. With exception of the NS1 ELISA, a use of the mentioned systems as accompanying marker test is possible keeping the limitations of each system in mind.

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DEVELOPMENT OF A REAL-TIME PCR ASSSAY BASED ON PRIMER-PROBE ENERGY TRANSFER (PriProET) FOR THE SENSITIVE DECTECTION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS

Michael J. McMenamy[1], John McKillen[2], Bernt Hjertner[1], Brian M. Adair[2], Gordon Allan[2]

[1]Department of Veterinary Science, The Queen's University of Belfast, Belfast, Northern Ireland BT43SD, [2]Veterinary Sciences Division, Agri-Food and Biosciences Institute, Belfast, Northern Ireland BT4 3SD

Keywords: ILT Real-Time, PriProET

Introduction and objectives

The avian Alpha Herpesvirus ILTV can be responsible for respiratory infections in poultry. The resultant symptoms can be confused with other respiratory infections such as Avian Influenza (AIV). It is therefore prudent to devise a straight-forward test capable of screening multiple potentially infected samples in real-time in efforts to detect ILTV. One potential method is to use the two fluorophore based system Primer-Probe Energy Transfer (PriProET).

PriProET assays are characterised by one fluorophore located on the 5' end of one of the primers (the donor fluorophore) with another fluorophore located on the 3' end of the probe (acceptor fluorophore) (Fig 1.)[3]. The maximum distance between acceptor and probe is ~40 nucleotide. During amplification primers will extend and the probe anneals to its target and energy transfer occurs between donor and acceptor fluorophores, permitting quantification of a specific target. As with other real-time PCR systems, PriProET utilises melt curve analysis. In the case of PriProET the melting profile data is in direct relation to the hybridisation efficiency between probe and target. Thus mismatch discrimination based on melt curve analysis is achievable. Here we describe the application of this PriProET to the sensitive detection of Infectious Laryngotracheitis virus (ILTV).

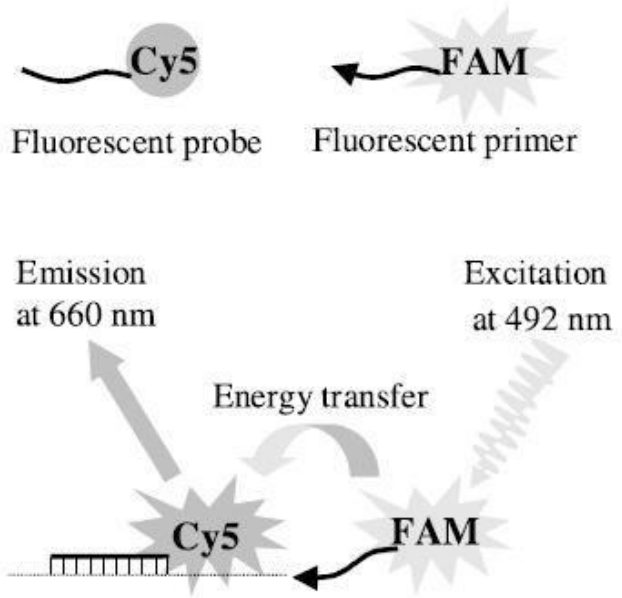

Fig 1. Energy transfer from donor fluorophore to acceptor fluorophore permitting real-time detection of resultant fluoresence apparent in PriProET based assays.

Materials and methods

Primer and probe sequences were designed with the assistance of Primer 3[1] and OligoAnalyzer 3.1[2] on-line software. A 5' FAM labelled forward primer, 3' Texas Red labelled probe and an unlabelled reverse primer were purchased from Sigma-Aldrich (Haverhill, Suffolk, UK). The assay target region was situated in a conserved area of the Thymidine Kinase gene and selection was based on a Genedoc alignment of 25 ILTV sequences. Assay optimisation and testing was carried out using a PCR amplicon spanning the Thymidine Kinase gene amplified from Solvay ILTV Vaccine (Fort Dodge). Thymidine Kinase amplicon fragment sequence was confirmed by sequencing with BigDye Terminator v3.1 (Applied Biosystems, Cheshire, UK). PCR product was spectrophotometrically quantified and then titrated in dilutions of known copy number. Dilutions ranging from $2x10^9$ to $2x10^0$ copies/µl were used for sensitivity testing. The PriProET reaction was carried out according to the methods of Rasmussen *et al.*[3], utilising 5'-*exo*$^-$ Titanium *Taq* polymerase from Clontech (Saint-Germain-en-Laye, France) which prevents the labelled probe from being degraded. 55 cycles of real-time PCR were carried out on a Roche LC480 instrument (Burgess Hill, UK) in 25µl reaction volumes containing 2µl of template. Optimisation involved running gradients of annealing temperature against Mg^{2+} concentration.

Results

Optimisation produced a PriProET assay designed to work with a Ta of 60°C, and 4mM Mg^{2+} and primer concentrations of 0.3µM for the labelled primer, 0.2µM for the unlabelled primer and 0.3µM for the probe. Sensitivity testing showed the assay capable of detecting an ILTV derived amplicon target at $2x10^1$ copies/µl. Fig 2. shows a standard curve for titrated ILTV vaccine strain Solvay Thymidine Kinase gene amplicon.

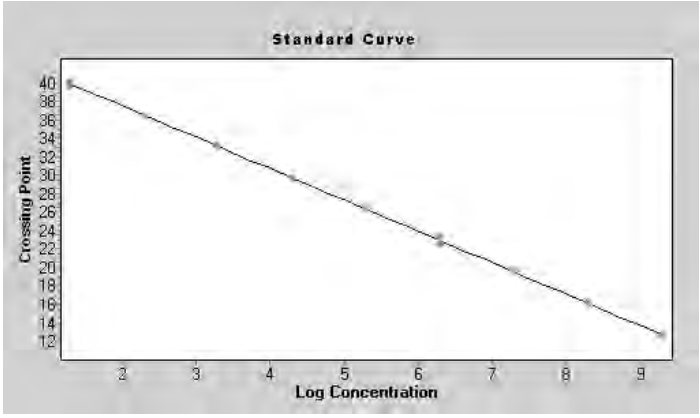

Fig 2. Standard curve for crossing point vs. log concentration for the ILTV PriProET assay with dilutions of standard DNA from $2x\ 10^9$ to $2x\ 10^1$ copies/µl.

Discussion and conclusions

PriProET offers a rapid and effective method of detecting ILTV in real-time. In the case of this assay detection from start to finish including time required to extract a sample for amplification (using the automated MagNa Pure LC nucleic acid extraction system from Roche) could be as little as three and a half hours. A potential advantage over other real-time methods is that PriProET permits melting curve analysis post amplification, providing additional information regarding the homology of the probe to the target. Although ILTV has a relatively well conserved genome, PriProET can be utilised to discriminate between vaccine-like and virulent isolates. Alternatively PriProET may be useful for the detection of less well conserved viruses because of the robustness demonstrated by the assay which is evident in the probe's ability to compensate for multiple mismatches in the target sequence. This ILTV PriProET assay has been shown to be capable of sensitive detection of ILTV to 10^1 genome equivalents. Further work including specificity testing on related avain viruses and on ILTV clinical samples is currently ongoing.

Acknowledgements

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DEVELOPMENT OF AN ACCELERATED LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAY FOR SENSITIVE DECTECTION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS

Michael J. McMenamy[1], John McKillen[2], Bernt Hjertner[1], Brian M. Adair[2], Gordon Allan[2]

[1]*Department of Veterinary Science, The Queen's University of Belfast, Belfast, Northern Ireland BT43SD;*
[2]*Veterinary Sciences Division, Agri-Food and Biosciences Institute, Belfast, Northern Ireland BT4 3SD*

Keywords: ILTV, isothermal, LAMP

Introduction and objectives

ILTV is an avian Alpha Herpesvirus, originally characterised in chickens and responsible for respiratory infections that may present symptoms similar to Avian Influenza (AIV). It is therefore essential to develop a rapid ILTV assay that can permit differentiation from AIV.

Accelerated Loop-mediated Isothermal Amplification (LAMP) is an isothermal (63-65°C) amplification method that utilises the strand-displacement activity of *Bst* DNA polymerase Large Fragment to amplify DNA specifically and rapidly through auto-cycling of a turnover structure possessing stem-loops at either end. A basic LAMP reaction will utilise four different primers specifically targeting six different regions of a target sequence. Accelerated LAMP employs an additional two "Loop-primers" to increase the number of starting points for DNA amplification via the LAMP method and thus decreasing the time required to achieve amplification[1]. Amplification and detection is achieved in a single tube format with DNA being amplified 10^9-10^{10} times in around 15-60 minutes[1]. Due to the use of Bst DNA polymerase there is no requirement to denature the target DNA thanks to its strand-displacement properties. This permits reactions being carried out it a laboratory waterbath at manageble temperatures. Under optimal conditions LAMP reactions can result in the precipitation of magnesium pyrophosphate which can be easily seen with the naked eye. Alternatively gel electrophoresis can be used to visualise products or even SYBR green to view the reaction in real-time. Here we describe the application of this isothermal amplification technique to the sensitive detection of Infectious Laryngotracheitis virus (ILTV).

Materials and methods

The six oligos required for ILTV accelerated LAMP were supplied by Sigma-Aldrich (Haverhill, Suffolk, UK). Assay design utilised dedicated software (Primer Explorer V.4) provided by Eiken Chemical company (Tokyo, Japan).The assay target region was situated in a conserved area of the Thymidine Kinase gene and selection was based on a Genedoc alignment of 25 ILTV sequences. Assay optimisation and testing was carried out using a PCR amplicon spanning the Thymidine Kinase gene amplified from Solvay ILTV Vaccine (Fort Dodge). Thymidine Kinase amplicon fragment sequence was confirmed by sequencing with BigDye Terminator v3.1 (Applied Biosystems, Cheshire, UK). PCR product was spectrophotometrically quantified and then titrated in dilutions of known copy number. Dilutions ranging from $2x10^9$ to $2x10^0$ copies/µl were used for sensitivity testing. The LAMP reaction was modified from the methods of Nagamine *et al.*[2] and Notomi *et al.*[3]. Reactions were carried out in 25µl volumes containing 0.2µM each of outer primers, 1.6µM each of inner primers, 0.8µM each of loop primers, 1.4mM dNTP's, 0.8M Betaine, 20mM Tris-HCl (pH8.8), 10mM KCl, 10mM $(NH_4)SO_4$, 8mM $MgSO_4$, 0.1% Tween 20, 16 units Bst DNA polymerase (New England Biolabs Inc., MA, US) and 2µl of DNA target sample. Reactions were allowed to proceed at 65°C for up to 90 minutes in a DYAD DNA Engine Thermal Cycler (MJ Research, MA, US) before inactivating the *Bst* polymerase at 80°C for 20 minutes. Reaction products were then analysed by gel electrophoresis.

Results

To test the detection limit of the ILTV LAMP assay titrations of the Thymidine Kinase gene from 10^9 to 10^0 copies per µl were tested in 3 separate experiments, each increasing by 30 minute increments up to 90 minutes. After 30 minutes (Fig 1.) the assay was capable of detecting 10^6 copies of the Thymidine Kinase gene target material. At 60 minutes (Fig 2.) the assay detected 10^2 copies of target. However, after 90 minutes the assay had not detected beyond 10^2 copies of genome equivalents.

Discussion and conclusions

Accelerated LAMP offers a rapid and specific method of amplifing ILTV in efforts to discriminate between it and other more severe respiratory viral infections in poultry. In the case of this assay the duration of the accelerated LAMP reaction was limited to 90 minutes to demonstrate the assay's effectiveness and speed in comparison to that of standard PCR based detection chemistries. Another advantage over traditional thermal real-time methods is that the use of six primers targeting eight specific regions of a viral genome in accelerated LAMP ensures the amplification of a unique target thus limiting the chance of any non-specific amplification that may occur when using a primer/probe based system. Also, accelerated LAMP requires little specialised equipment thus potentially permitting it to be carried out in the field. This ILTV accelerated LAMP assay has been shown to be capable of rapid and sensitive detection of ILTV to 10^2 genome equivalents after just 60 minutes. Further work including specificity testing on related avain viruses and on ILTV clinical samples is currently ongoing.

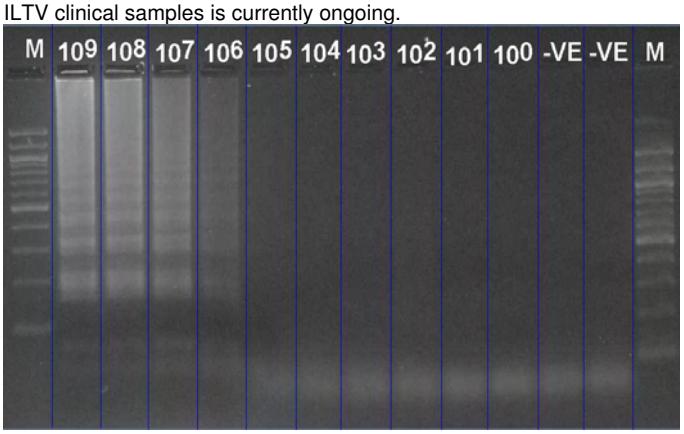

Fig 1. ILTV LAMP detection of Solvay ILTV Vaccine Thymidine Kinase gene amplicon. LAMP patterns from left to right represent LAMP assay detection limits after 30 minute reaction.

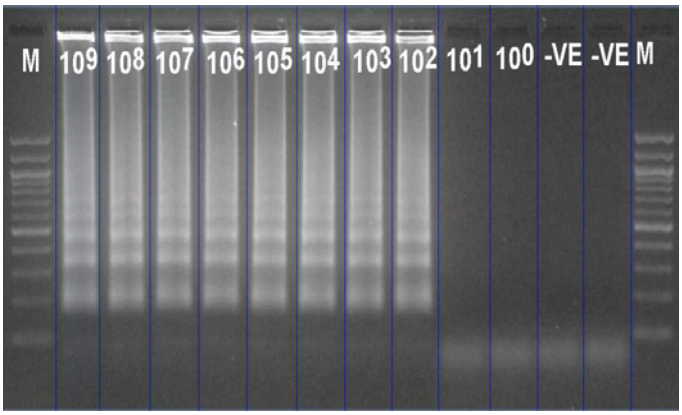

Fig 2. ILTV LAMP detection of Solvay ILTV Vaccine Thymidine Kinase gene amplicon. LAMP patterns from left to right represent LAMP assay detection limits after 60 minute reaction.

Acknowledgements

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TOWARDS A JEMBRANA DISEASE VIRUS SPECIFIC DIAGNOSTIC IMMUNOASSAY - PEPTIDE MAPPING OF GAG AND ENV PROTEINS OF BOVINE LENTIVIRUSES

Tegan J. McNab[1], Moira Desport[1], I.W. Masa Tenaya[1,2], Nining Hartaningsih[2], Graham E. Wilcox[1]

[1]School of Veterinary and Biomedical Sciences, Faculty of Health Sciences, Murdoch University, Perth, Australia;
[2]Disease Investigation Centre, Region VI, Denpasar, Indonesia

Keywords: bovine immunodeficiency virus, epitope mapping, Jembrana disease virus, synthetic peptides.

Introduction and objectives

Serological evidence for the presence of two bovine lentiviruses, Jembrana disease virus (JDV) and bovine immunodeficiency virus (BIV) has been reported in the Bali cattle population of Indonesia[1]. JDV causes an acute disease in Bali cattle (*Bos javanicus*) after a short incubation period. Disease is characterised by a high plasma viraemia, fever and a case fatality rate of 20%[4]. The virus has since become endemic throughout Bali, Java, Sumatra and Kalimantan. In contrast BIV infection of *Bos taurus* cattle does not appear to result in an overt clinical disease. Serological studies have reported a worldwide distribution of BIV.

Due to cross-reacting epitopes, infections with these viruses cannot be differentiated by current serological tests using native or recombinant BIV or JDV antigens[3]. Antigenic cross-reactivity has been demonstrated in the capsid (Ca)[4], matrix (Ma)[3] and the transmembrane (Tm) glycoprotein[2].

In Indonesia, ELISA using a JDV Ca recombinant antigen produced in *E. coli* is currently used to diagnose infections with JDV. This assay does not distinguish between JDV and BIV infections and whilst PCR-based tests offer specificity, in a resource-limited region such as Indonesia these are not practical. JDV and BIV proviral DNA is also not reliably detected in recovered cattle under field conditions.

The aim of this study was to precisely map the epitopes in Gag and SU to identify an epitope(s) that will differentiate between JDV and BIV infections in a serological assay. Differential peptides against which a strong humoral response can be reliably detected will then be tested in Indonesia for specific serological diagnosis of JDV infections.

Materials and methods

To precisely map linear epitopes in the JDV Ma, Ca and SU, a pepscan analysis was performed using 155 synthetic peptides. Overlapping peptides were synthesised to cover the entire amino acid sequence of Ma, Ca and SU of $JDV_{TAB/87}$ (Mimotopes). The peptides were 16 amino acids long and overlapped by 11 residues. A synthetic peptide ELISA was then optimised to test serum samples. A panel of sera taken from experimentally infected cattle was used in this study (n = 15). Serum was taken between 71 and 138 days post JDV infection (n = 12) or 103 days post BIV infection (n = 3). Hyperimmune serum from each infection was also tested. Samples were seropositive when tested with ELISA using recombinant JDV Ca or BIV CA or a transmembrane peptide.

Results

A large proportion of the Ma peptides were immunoreactive (21/25). Both JDV and BIV sera reacted to a large number of peptides. BIV sera reacted to numerous peptides throughout Ca (Fig. 1. bottom). Surprisingly, JDV sera reacted to considerably less (Fig. 1. top and middle). The opposite was seen in SU where a number of peptides reacted with JDV sera while few peptides reacted with BIV sera.

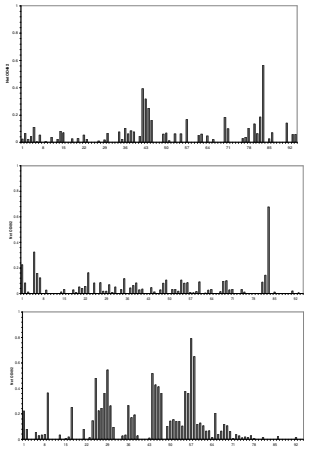

Fig. 1. Pepscan analysis of overlapping peptides corresponding to the Ma and Ca proteins and a portion of the SU protein. Represented are the pepscans performed with pooled sera taken 93 and 98 days post JDV infection (top), JDV hyperimmune serum (middle) and BIV hyperimmune serum (bottom). Peptide numbers 1 to 25 span Ma, 26 to 71 span Ca and 72 to 94 span the start of SU.

Discussion and conclusions

These peptides provide potential reagents for the identification of bovine lentivirus infections as well as reagents suitable for differentiating between the 2 infections. Peptides such as those in SU (peptide number 83) would be useful in differentiating BIV and JDV infections while those in Ma would be useful in identifying bovine lentivirus infections. Rigorous testing of samples taken from natural infections will be undertaken to evaluate the usefulness of these peptides in differentiating between JDV and BIV in a field situation. JDV hyperimmune serum failed to recognize peptides located in Ca, even within the major homology region (spanning peptides 52-59). This serum does however react with both JDV and BIV recombinant Ca proteins suggesting that antibodies recognize conformational epitopes which can't be identified by synthetic peptides.

Acknowledgements

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GENETIC CHARACTERIZATION OF CHICKEN PARVOVIRUS STRAINS FROM NATURALLY INFECTED HUNGARIAN FLOCKS

Elena Alina PALADE[1], Ákos HORNYÁK[2], Zoltán DEMETER[1], Mihály DOBOS-KOVÁCS[1], Zsófia BENYEDA, Miklós RUSVAI[1], János KISARY[3]

[1]Department of Pathology and Forensic Veterinary Medicine, Faculty of Veterinary Science, Szent István University, Budapest, Hungary;
[2]Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Budapest, Hungary;
[2]RecombiVet Kft, 2085. Pilisvörösvár, Kápolna u. 143b, Hungary

Keywords: chicken parvovirus, phylogeny, viral enteric disease

Introduction and objectives

The viral enteric disease complex is a serious economic problem in the poultry industry. In broiler chickens the major enteric disease complex is known as runting-stunting syndrome (RSS). It is characterized by diarrhoea, depression, ingestion of litter, increased vocalization and huddling. Morbidity and mortality are variable, and the economic impact from enteric disease is primarily due to poor production, failure of affected birds to grow, as well as increase in costs of therapy, and poor feed conversion efficiency. Viruses from numerous families have been identified in the intestinal tracts of poultry with enteric disease: *Astroviridae*, *Coronaviridae*, *Reoviridae* and *Rotaviridae*. Kisary and his co-workers demonstrated by electron-microscopy (EM) the presence of parvovirus-like particles in intestinal homogenates of 10 days old chickens with enteric disease (Kisary et al., 1984) and by inoculating 1 day old chickens with the purified viral particles obtained the characteristic clinical signs of RSS (Kisary, 1985). The following molecular biological studies revealed that the virus belongs to the *Parvoviridae* family (Kisary et al., 1985a). A recent survey revealed the presence of parvovirus infection in chicken and turkey samples from 8 different states in the USA (Zsak et al., 2009). Chicken parvovirus (ChPV) infection was demonstrated by EM and polymerase chain reaction (PCR) technique in five naturally infected Hungarian broiler flocks experiencing diarrhoea, increased mortality and poor feed conversion efficiency.

Materials and methods

Sample collection, Histopathology and EM

Carcasses from 5 Hungarian broiler flocks experiencing increased mortality were sent to the Department of Pathology and Forensic Veterinary Medicine (Szent Isván University, Budapest, Hungary) for diagnostic purposes. Tissue samples from various organs (thymus, spleen, intestine) were stored in 8 % neutral buffered formaldehyde solution for histological examination and routinely processed. Intestinal content samples were suspended 1:3 in distilled water, cleared by low speed centrifugation, followed by 20 min at 9000 x g. The samples were prepared according to the single-droplet negative staining technique and examined at a transmission electron microscope (JEOL, Japan).

PCR and Phylogeny

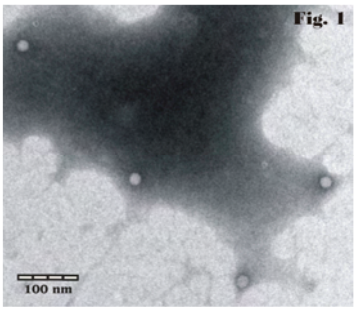
Fresh tissue samples were homogenized in 2 ml sterile phosphate-buffered saline. The viral DNA was purified from the supernatants and amplifications were performed in a continuous polymerase chain reaction method. The 50 µl reaction mixtures contained sterile deionised water, 10X PCR buffer without $MgCl_2$, 2 mM dNTP mix, 25 mM $MgCl_2$ with a final concentration of 1-4 mM, 0.8 µM of the forward and reverse primers PVF1, PVR1, previously described by Zsak et al. (2009), Taq DNA Polymerase, 1.25 u/50 µl, 1 µl (Fermentas, Lithuania) and 2.5 µl of template DNA. The reactions were performed in a PCR Sprint Thermal Cycler SPRT001 (Hybaid, UK). Following PCR, the amplicons were electrophoresed in a 0.5X Tris borate-EDTA-agarose gel, 1.2% SeaKem® LE Agarose, (Cambrex, USA) at 80 V for 80 min. The gel was stained with ethidium bromide (10 µg/µl) and the bands were visualized by UV translumination at 312 nm using a TFX 35M UV transluminator (Life Technologies, UK) and photographed with an Alpha Digidoc RT2 Documentation and Analysis System (Alpha Innotech, USA). Product sizes were determined with reference to a 50 bp molecular weight ladder (Fermentas, Lithuania). Following electrophoresis the amplicons were cut out from the gel and DNA was extracted with the QiaQuick Gel Extraction Kit (Qiagen, Germany). Fluorescence-based direct sequencings were performed in both directions on the amplicons at the Biomi Kft (Gödöllő, Hungary) and Biogon Kft (Budapest, Hungary) employing an ABI 3100 genetic analyzer (Applied Biosystems, USA). Nucleotide sequences were identified by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) search, against GenBank databases. Nucleic acid sequences were compiled and aligned using the Align Plus 4 software (Scientific & Educational Software, USA). Phylogenetic analysis was performed with the help of the ClustalX program. Phylogenetic tree of the nucleic acid sequences was established by using sequence data of the Hungarian ChPV strains and the only two existing ChPV strains retrieved from the GenBank. Blocks of sequence data leading 524 bp were used for the analysis. The phylogenetic tree was constructed by neighbourjoining with a two-parameter distance matrix using the Phylip program.

Results

The chickens from all five flocks presented stunted growth and evident signs of enteritis. Histological examination revealed lymphocyte depletion in the spleen and thymus, and acute enteritis in the small intestine. The EM examination revealed the presence of numerous icosahedral, non-enveloped viral particles, measuring about 22 to 25 nm in diameter (Fig. 1). Based on their ultrastructural morphology, the viral particles were identified as members of the *Parvoviridae* family. The PCR resulted in the successful amplification of a 561 bps product in case of all five samples. The alignment of the nucleic acid sequences of the Hungarian strains revealed a high level of identity compared to the two ChPV sequences existing in the GenBank (95.8 to 98.8 % compared to the turkey isolate and only 95.0 to 96.9 % compared to the chicken isolate). The homology level among the Hungarian strains varied from 96.0 to 98.6 %). The phylogenetic tree constructed based on the nucleic acid sequence of the analyzed segment revealed that the Hungarian strains were more closely related to the turkey isolate than to the chicken isolate (Fig. 2).

Discussion and conclusions

Our investigations revealed that the scarcely known ChPV is present in the analyzed Hungarian flocks experiencing RSS. The presence of the pathogen was demonstrated using EM and a highly specific PCR-based method. The nucleic acid sequence analysis revealed that the Hungarian strains are closely related among themselves. The phylogenetic analysis has revealed that the Hungarian strains are more closely related to a previously described ChPV strain of turkey origin, than to the also described chicken isolate (Fig. 2). This finding seems to emphasize a potentially major role of turkey flocks in the epidemiology of ChPV. On the other hand, the exact role of ChPV in the RSS should be more closely studied, since more pathogens are suspected to be involved in the pathogenesis of this disease complex.



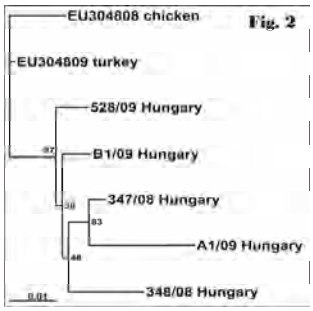


Fig. 1: Transmission electron micrograph showing viral particles characteristic for Parvoviridae family.

Fig. 2: Phylogenetic relationship of the investigated Hungarian ChPV strains, and the 2 sequences retrieved from the GenBank. Bar on the left demonstrates the genetic distance.

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A BACULOVIRUS EXPRESSED NS3 PROTEIN OF BOVINE VIRAL DIARRHEA VIRUS DISPLAYS CONFORMATIONAL AND ANTIGENICAL PROPERTIES AS THE NATIVE PROTEIN

Giulia PEZZONI, Emiliana BROCCHI

Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna, Brescia Italy

Keywords: baculovirus-NS3, BVDV, monoclonal antibodies

Introduction and objectives

One of the major immunological proteins of pestiviruses is the nonstructural protein 3 (NS3), a multifunctional enzyme with at least two domains associated with enzymatic activities: a serine protease activity and a NTPase-elicase activity. Antibody to NS3 are detected in animals infected with all pestivisuses. This study describes the expression of the entire sequence of NS3 (rNS3) and its NTPase-elicase domain (rNS3E) in a baculovirus system and the antigenic characterisation of the recombinant products, with the final objective to use them as source of antigen in serological assays for pestiviruses.

Materials and methods

Expression of the recombinant protein. The RNA corresponding to NS3 (aa1680-2362) and NS3-NTPase-elicase domain (aa1852-2362) of BVDV NADL strain were cloned in the baculovirus expression vector pFastBacHT. The recombinant baculoviruses expressing the two proteins were obtained by Bac to Bac system (Invitrogen). The proteins were produced in Sf9 cells (Spodoptera frugiperda 9) infected with the respective recombinant baculovirus; after three days of infection the cells were harvested and lysed in a non-denaturing buffer.

Monoclonal Antibodies (MAbs). A panel of three anti-NS3 MAbs (3H4, 3A3, 1F2) previously raised against an Italian isolate of BVDV (Brocchi et al.) were used for NS3 characterisation.

Immunofluorescence (IF). IF was carried out on Sf9 cells infected with recombinant baculoviruses, fixed with acetone and processed with dilutions of the three MAbs.

Western-blotting. Lysates of Sf9 infected with recombinant baculoviruses were separated by SDS-PAGE and electrophoretically transferred onto PVDF membranes. These were incubated with positive and negative sera and with MAbs.

Sandwich ELISA. The three MAbs were used as catching and peroxidase-conjugated antibody to evaluate the capability to bind the recombinant proteins (crude lysates) as well as the viral (BVDV) antigen.

Competitive ELISA. The above sandwich ELISAs were modified in competitive assays. Each MAb or immune sera were individually incubated with Mab-captured antigens simultaneously with each peroxidase-conjugated MAb. This enabled the evaluation of MAbs and sera reciprocal competition.

Ag-capture ELISA. The recombinant antigens trapped by each coated MAb were reacted with representative positive and negative bovine sera; bovine immunoglobulins bound to antigens were then detected with an anti-bovine IgG conjugated to peroxidase.

Results

Optimized conditions to obtain high yields of the recombinant proteins rNS3 and rNS3E were molteplicity of infection 10 and harvest after three days post-infection.

Both recombinant antigens were recognized by the three MAbs in IF, but reacted in western blotting only with polyclonal, positive sera. Actually, the three MAbs are directed against conformational epitopes (1), that are missed in denatured proteins, while positive sera bind also to linear epitopes.

The reactivity profiles in ELISA sandwich of recombinant and native antigens were equivalent (table 1); differences in signal intensity are to be attributed to better yields of recombinant products than of viral antigen. No signal was observed using the same MAb as catcher and conjugate, since the epitopes are present in a single copy on the proteins. rNS3 and rNS3E were efficiently captured by each MAb, but 3H4 is the best catching MAb for both viral and recombinant antigens.
Competition profiles reported in figure 1 showed that the three anti-NS3 MAbs do not compete each other, proving that they recognize three different, independent epitopes, while positive control sera competed at 100% level against each MAb.

Both recombinant antigens, captured by any of the three MAbs, exposed epitopes that were easily and efficiently recognized by pestivirus-positive sera (figure 2).

Discussion and conclusions

Two recombinant NS3 proteins were successfully produced in a baculovirus-system and their antigenic properties were compared with those of the native BVD viral antigen.

Both recombinant proteins were recognised by monoclonal antibodies specific for three different conformational epitopes, as well as by BVDV-positive sera. These reactivities provide evidence that the recombinant proteins obtained reproduce folding and antigenicity of the native viral proteins, providing the conditions for the development of a functional antibody-detection ELISA.

Table 1. Comparative performance of MAbs-based sandwich ELISA using rNS3, rNS3E or BVD virus

		Peroxidase conjugated MAbs								
		3H4	3A3	1F2	3H4	3A3	1F2	3H4	3A3	1F2
Catch. MAbs	3H4	+/-	++++	+++	-	+++	+++	-	+++	+++
	3A3	++++	+/-	+++	+ +	-	++	+ +	-	+/-
	1F2	+++	+++	+/--	++	++	-	+/-	-	-
		rNS3			rNS3E			BVD Viral antigen		

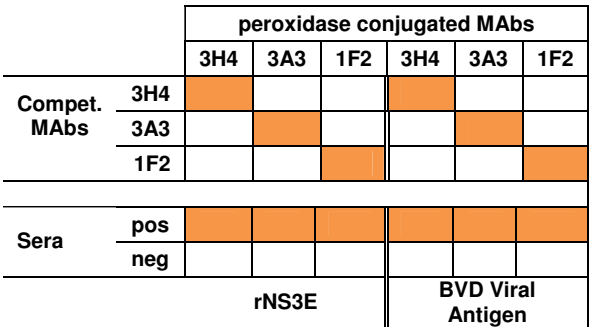

Figure 1. Competition profile of MAbs and immune sera for binding to rNS3E or to BVD viral antigen (rNS3 showed the same profile)

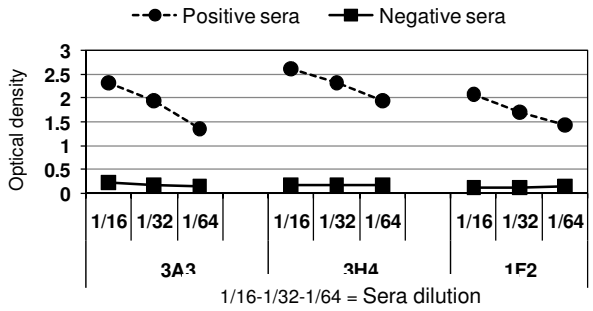

Figure 2. Reactivity in Ag-capture ELISA of positive and negative sera toward the recombinant NS3E trapped by each MAb (rNS3 showed the same profile)

Acknowledgements

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FIRST EVIDENCE OF *CAPRINE HERPESVIRUS 1* IN GOAT HERDS IN PIEDMONT, ITALY

Monica PITTI[1, 2], Antonia SCIARRA[1], Mariangela ANDRÀ[1], Pasquale AMATO[1], Giuseppe RU[1], Loretta MASOERO[1]

[1] *Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Torino, Italy;*
[2] *Faculty of Veterinary Medicine, Università degli studi di Milano, Italy*

Keywords: CpHV-1, ELISA, seroprevalence

Introduction and objectives

Caprine herpesvirus 1 (CpHV-1) belongs to the *Herpesviridae* family, *Herpesvirinae* subfamily; it is responsible of a letal sistemic disease in 1-2 week old kids and of vulvovaginitis, balanopostitis and abortion in adult goats (5).

The infection is present worldwide and the prevalence of the disease is higher in countries where goat breeding is common: more than 50% in Greece (1) , 30-60% in southern Italy (3) and 21% in Spain (1).

CpHV-1 belongs to a cluster of alphaherpesviruses of ruminants including Bovine Herpesvirus 1 and 5 (BoHV-1, BoHV-5), CpHV-1, Cervine Herpesvirus 1 (CerHV-1) e Rangiferine Herpesvirus 1 (RanHV-1) (2). This antigenical similarity may create difficulties particularly with serological diagnosis of IBR (4).

Currently the serological diagnosis of IBR is based on the use of commercial BoHV-1 glycoprotein B (gB) and glycoprotein E (gE) blocking enzyme-linked immunosorbent assays (ELISA); gB is an envelope glycoprotein well conserved in all alphaherpesviruses of ruminants (4). Previous studies on BoHV-5 e CpHV-1 showed that ELISA-gB test allows to identify antibodies against ruminants' alphaherpesviruses, whereas ELISA-gE test is positive only in case of BoHV-1 infection: it has been suggested that the use of both tests allows the discrimination of BoHV-1 infection and other alphaherpesviruses of ruminants (4)(5).

The aim of this study is the evaluation of the presence of CpHV-1 in goat herds in Piedmont and a preliminary estimate of the seroprevalence of the infection.

Materials and methods

We analysed goat sera sent to the Laboratory of Virology of the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta in Torino (Italy).

A convenience sample of 2,041 field sera was collected during the period June 2008 – March 2009 in 151 goat herds dislocated in 8 provinces of Piedmont: Torino [63 herds], Novara [46], Verbania [25], Vercelli [7], Asti [4], Biella [3], Alessandria [2] and Cuneo [1]. The sera were sent to the laboratory for various serological examinations.

To detect anti-gB antibodies we used the HerdCheck IBRgB Competition ELISA kit [Idexx Laboratories].

Positive sera were tested to detect antibodies against gE using the HerdCheck IBRgE Competition ELISA kit [Idexx Laboratories].

The sample size per herd was large enough to detect, with a 95% confidence level, the presence of the disease if the prevalence was higher than 2.91%.

Considering a prevalence expected value of 50%, at a confidence level of 95%, the estimate obtained with this sample is associated to an absolute error of 1.94%.

We calculated the within-herd prevalence in positive herds where all goats have been tested.

In most of the negative herds all animals have been checked; in herds, where only a group of goats have been tested, we evaluated whether the sample could reveal the lowest prevalence observed in positive herds.

Results

A first screening test to detect anti-gB antibodies was made: 322 samples (15.8%) resulted positive and 1,719 negative. The 322 gB-positive samples were tested to detect anti-gE antibodies: 15 samples were positive, 16 doubtful and 291 negative and therefore attributable to CpHV-1.

Individual seroprevalence was 14.3% (CI95%: 12.7-15.8%).

The positive herds were 47 out of 151 leading to a herd-level prevalence of 31.1% (CI95%: 23.7-38.5%).

In the 24 herds totally tested, the within-herd prevalence ranged from 4.8% to 68.4%, the median is 25%.

In 77 negative herds all goats have been tested whereas in 27 herds only a part of them. Evaluating the number of tested animals, we calculated the maximum prevalence compatible with a negative result obtained in the tested animals. In 26 herds out of 27 the sample size could not have been sufficient to detect a prevalence of 4.8% (with a 95% confidence level), the lowest value found.

Discussion and conclusions

The current study shows that in the Piedmont Region the CpHV-1 infection is present involving about one third of the herds with a wide range on within-herd prevalence rates.

The detection of antibodies against ruminants' herpesviruses in goat sera indicates the presence of CpHV-1 in the monitored herds with a regional individual seroprevalence lower than those observed in Spain (22%)(1) and in Corsica (58%) (4). The 31% herd-level prevalence we observed is consistent with the prevalence calculated in southern Italy (3) whereas higher than the value obtained in Spain (1) and lower than the prevalence of the disease in Greece and Corsica (4). The within-herd prevalence (range:17,4-60,0) is lower than the one verified in Corsica (range:38,7-73,7) (4).The use of a convenience sample allows to make a preliminary estimate of the prevalence of the disease and there's no particular reason to suppose that the sampling criteria could have biased our results.

The detection of seropositivities prompts us to plan a wider screening of goat herds in Piedmont. A representative sample will be tested in order to calculate the true prevalence of CpHV-1 in Piedmont, to verify the presence of clusters of infection and to characterize the clinical patterns of the disease.

An open problem is still the accuracy of the diagnostic protocol. A previous study reported, sensibility and specificity values of the ELISA-gB kit of 100% and 93.5% respectively (based on data obtained from experimentally infected goats)(4). We will set up methods in order to confirm the presence of CpHV-1 in seropositive herds and to assess the accuracy of the diagnostic approach.

The description of the epidemiological features of the disease, the evaluation of its economical impact on herd production and an effective diagnostic protocol are essential for programming a regional monitoring and control plan in the next future.

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PYROSEQUENCING ANALYSIS FOR A RAPID FOWL ADENOVIRUS SPECIES CLASSIFICATION

Matteo Samuele PIZZUTO, Cristian DE BATTISTI, Sabrina MARCIANO, Ilaria CAPUA, Giovanni CATTOLI

Istituto Zooprofilattico Sperimentale delle Venezie, OIE/FAO and National Laboratory for Avian Influenza and Newcastle Disease, Legnaro (PD), Italy

Keywords: hexon, fowl adenovirus, pyrosequencing

Introduction and objectives

Fowl adenoviruses (FAdVs), belonging to the *Aviadenovirus* genus of the family *Adenoviridae,* are endemic worldwide and known to cause inclusion body hepatitis, quail bronchitis, hydropericardium syndrome and pancreatic necrosis (1).

FAdV classification is very useful for epidemiological tracing and is of critical importance where vaccination is to be used for the control of the disease. FAdVs have been grouped into 5 species based on their molecular structure and then divided into 12 serotypes, based largely on cross-neutralization assays (1).

The hexon protein is the major capsid protein of the non-enveloped virion on which type-, group-, and subgroup-specific determinants are located. The hexon consists of conserved regions (pedestals), which are located inside the virion, and the variable loops, which protrude from the surface containing the type-specific neutralizing epitopes. Due to this interaction with the immune system, the sequence identity between loop regions of different species is low (2).

Several investigations have reported the use of polymerase chain reaction (PCR) for the detection of hexon adenoviral DNA in combination with the restriction enzyme polymorphism (RFLP) or conventional DNA sequencing in order to differentiate the species (3-5).

RFLP as well as conventional DNA sequencing approaches are time consuming, often require extensive interpretation, and the running cost per analysis can be relatively expensive to be used as routine typing tool. For this reason, a rapid Fowl Adenovirus species classification, based on 30bp of the hexon loop L1 sequence, has been set up using Pyrosequencing technique.

Materials and methods

Fowl adenoviruses, representative of the currently recognized distinct serogroups, were grown in chicken embryo liver cells obtained from 14-day-old specific pathogen free embryos (Lohmann). Cell monolayers were inoculated and incubated for 2 to 3 days, until an intensive cytopathic effect could be seen.

The infected cells and their supernatants were then harvested and frozen and thawed for three times. The suspension was centrifuged at 3000 *g* to eliminate cell debris and the supernatant was stored at –20°C.

Hexon L1 loop Genbank sequences were aligned using MEGA software (version 4.1) in order to find a 30bp region capable to discriminate among different FAdV species. The consensus sequence was used to design PCR forward and Pyrosequencing primer: FAdV-Pyro-fw.

After adenoviral dsDNA extraction (Roche High Pure PCR Template Preparation Kit) a PCR was performed using the couple of primers: FadV-Pyro-fw and Biotinylated Hexon B-rev, the latter previously published (5). Hexon L1 loop PCR products (765bp) were sequenced in order to confirm the identity of the viruses. Biotinylated amplicons were purified, and the pyrosequencing reactions were carried out using a PyroMark ID platform pyrosequencer (Biotage). Amplification products were washed in a series of buffers, and single stranded, biotinylated DNA products were hybridized to FAdV-Pyro-fw sequencing primer. Pyrosequencing was performed using Pyro Gold reagents according to Biotage's recommendations.

Results

The primer set FadV-Pyro-fw / Biotinylated Hexon B-rev was used to amplify approximately 765 bp of the hexon gene from 21 different FAdVs. All the strains gave a clear amplification product without aspecific bands in 1 % agarose gel. Sequence analysis confirmed the hexon loop L1 amplification and the FAdV strains identity.

Pyrosequencing analysis of the PCR products, using FadV-Pyro-fw as sequencing primer resulted in good quality pyrograms up to 30 bp. The related sequences were analyzed and compared with a FAdVs hexon sequences library using IdentiFire software (Biotage). The results showed a perfect identification with the proper species (A-E) for all the viruses with scores ranging from 70% to 100% (Figure 1). On the strength of these results it was also possible to distinguish among different serotypes inside species A (CELO serotype 1), B (IBH-2A–TR22 serotype 5), D (A2-A serotype 9; 75 – 75-1-A – SR49 serotype 3; 685 – P7-A serotype 2) and E (CR119 serotype 6).

2009-05-12

IdentiFire

Summary Report

Biotage

Sample Id	Result	Score	
75-1A	D-Serotype 3	100	Details
C2B	C	100	Details
764	E	100	Details
506	C	100	Details
75	D-Serotype 3	100	Details
T8-A	E	100	Details
685	D-Serotype 2	100	Details
P7-A	D-Serotype 2	100	Details
58	E	95.3	Details
IBH-2A	B-Serotype 5	100	Details
B3A	E	100	Details
X-11-A	E	100	Details
A-2A	D-Serotype 9	100	Details
J2-A	C	100	Details
YR-36	E	95.3	Details
SR49	D-Serotype 3	100	Details
TR59	E	100	Details
KR5	C	100	Details
TR22	B-Serotype 5	69.1	Details
CR119	E-Serotype 6	100	Details
CELO	A-Serotype 1	100	Details
K- -BEADS + PS	No hits found.	0	Details
K- +BEADS + PS	No hits found.	0	Details
K- + BEADS -PS	No hits found.	0	Details

Figure 1. IdentiFire summary report.

Discussion and conclusions

Based on a short 30bp fragment of hexon L1 loop sequence, it is possible to sort the FAdVs in their proper species (A-E) and directly recognize several serotypes (1-2-3-5-6-9).

Starting from the PCR products the pyrosequencing analysis requires only 2 hours in order to obtain the final result, almost half of the time comparing with RFLP analysis or conventional DNA sequencing. Pyrosequencing analysis does not need any restriction or terminator enzyme; moreover the results are clearly reported as shown in Figure1.

This study provides a new approach for a rapid differentiation and classification of Fowl Adenovirus (FAdV) species using the pyrosequence analysis that is faster, less expensive and easier to interpret than RFLP or conventional DNA sequencing.

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DEVELOPMENT OF A COMPETIVE ELISA (C-ELISA) SYSTEM FOR THE DETECTION OF AVIAN INFLUENZA INFECTION IN SERA OF FIVE DIFFERENT AVIAN SPECIES

Michela RIGONI[1], Elisabetta VIALE[1], Marzia MANCIN[2], William DUNDON[1], Valeria BRASOLA[1], Calogero TERREGINO[1], Ilaria CAPUA[1]

[1]OIE/FAO and National Reference Laboratory for Newcastle Disease and Avian Influenza, OIE Collaborating Centre for Epidemiology, Training and Control of Emerging Avian Diseases, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padova, Italy;
[2]Public Health and Risk Analysis Department, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padova, Italy

Keywords: avian influenza, competitive ELISA, diagnosis

Introduction and objectives

Avian influenza (AI) is an important zoonotic disease with serious economic consequences. A global serological AI surveillance program is the key tool in the identification and control infection in birds. Serological tests used for AI antibody detection in poultry include the agar-gel immunodiffusion (AGID) test, hemagglutination–inhibition (HI) test and enzyme-like immunoassay (ELISA). ELISA tests have the advantage of screening large number of samples and giving rapid results, thus being ideal as a screening tool. However, the available commercial ELISA kits are only validated for use chicken sera and some turkey samples.

In the current study our laboratory, in the framework reference laboratory for Avian influenza, has developed and validated a competitive enzyme-like immunoassay (c-ELISA) for the detection of type A influenza antibodies in different avian species.

Materials and Methods

Viruses:

The H5N1 avian influenza virus (AIV) -A/chicken/Yamaguchi/07/04- was grown in SPF chicken embryonated eggs. The allantoic fluid was titrated and infectious titre expressed as embryos infective dose (EID50). The viral suspension obtained was used to immunize Balb-C mice to generate monoclonal antibodies (mabs) against the viral nucleoprotrein (NP).

Mabs: mabs producing hybridomas were generated following fusion of SpO2/Ag14 myeloma cells with splenocytes from immunised Balb-C mice. The specificity of mabs obtained was determined by comparing their reactivity in indirect-ELISA, immunofluorescence and western blotting.

The mab selected was than purified and conjugated with horseradish peroxidase (HRP) and tested in a c-ELISA assay (1).

c-ELISA validation: The c-ELISA assay was applied to a total of 1354 samples (662 positive and 692 negative), some obtained from 5 different species of birds (duck, chicken, turkey, ostrich, quail). All sera were treated as described in the OIE Manual (2).

Results

The efficacy of the test was compared with the haemagglutination inhibition (HI) assay and additionally with agar gel immunodiffusion AGID test (except for duck sera), following the OIE/UE guidelines (3).

The c-ELISA results correlated with the HI data by 99.3%, with sensitivity for duck, ostrich, turkey and chicken sera ranging from 95.21 to 100% and with specificity levels ranging from 93.59 to 100%.

The c-ELISA was unsuitable for quail sera due to low sensitivity and specificity.

Discussion and conclusions

In conclusion, the test appears to be a suitable tool for the screening of avian sera of chicken, turkey, duck and ostrich species. The results of our investigation indicate that the test is suitable for only 4 out of five species tested. This study also highlights the necessity of using fully validated tests, with particular reference to diseases such as AI which readily causes infection in multiple avian species.

Acknowledgements

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GENETIC CHARACTERIZATION OF CORONAVIRUSES IN SHELTER DOGS AND CATS IN LISBON

Ricardo C ROSADO, Ana DUARTE, Augusto BAPTISTA, Filomena OLIVEIRA, Ana MACHADO, Leonel FERNANDES, Luís TAVARES

CIISA, Faculty of Veterinary Medicine, Universidade Técnica de Lisboa

Keywords:, CCoV, coronavirus, FCoV, molecular epidemiology, viral detection

Introduction

Coronaviruses (CoV), members of the family Coronaviridae, are large, enveloped, single-stranded RNA viruses, responsible for disease in several species of mammals and birds. Clinical signs are usually enteric or respiratory, but can also be systemic. Presently they are classified into three different antigenic groups. Group 1 includes both canine (CCoV) and feline coronaviruses (FCoV) and group 2 includes the recently recognized canine respiratory coronavirus (CRCoV).

CCoV has been further classified into two genotypes, I and II, the first with high genetic similarity with FCoV. Both genotypes are responsible for the occurrence of enteritis in dogs, which can be fatal when associated in mixed infections with canine parvovirus (CPV), especially in younger dogs.

FCoV have different classifications according to genotype and biotype. Due to their serological and genomic features FCoVs are classified as types I and II, where type I is strictly feline while type II resulted from a recombination event between FCoV and CCoV.

FCoVs can be further classified into two biotypes. The enteric biotype (FECV) is present ubiquitously in cat populations, causing mild diarrhoea. FECV shedding can be transient, recurrent, or chronic over periods of months or years. The other recognized biotype of FCoV causes a lethal disease, feline infectious peritonitis (FIPV). This form with higher virulence results from genomic mutations consistently found in the 3c gene and only develops in a small percentage of animals, usually during primary infection and in kittens.

The emergence of human coronavirus (SARS) has incited renewed interest in coronaviruses, and serological and virological investigations have reported worldwide presence and prevalence of these viruses in both domestic, as well as in free-roaming stray or feral dogs and cats. This knowledge is especially relevant in kennel and animal shelters.

To investigate the genomic diversity of FCoV and CCoV in Lisbon's Municipal kennel, a virological survey was conducted which included canine distemper virus, canine and feline parvovirus, canine and feline coronavirus, feline immunodeficiency virus and feline leukaemia virus. All coronavirus positive samples were further characterized to assess the presence of different FCoV and CCoV genotypes within the animal population.

Materials and methods

Samples were collected from 49 dogs and 50 cats at the time of euthanasia, between October and November 2008 in the Lisbon Municipal Kennel. Faecal samples were collected by rectal swab, ressuspended in PBS and centrifuged prior to RNA extraction using QIAmp MiniElute Virus Spin Kit. Viral nucleic acids were kept at -80ºC until analysed.

In March 2009, 24 environmental swabs were collected from 6 occupied cells and cages randomly selected. The chosen cells were sampled every 4 days up to a total of 4 samples from each. These samples were suspended in PBS, centrifuged, and DNA/RNA was extracted following the same procedure as for the faecal swabs.

Dog samples and environmental swabs from dog cells were amplified by RT-PCR using two different pairs of primers for CCoV type I and type II (Pratelli et al., 2004). For detection of Feline Coronavirus, cat samples and environmental swabs from cat cages were assessed by RT-PCR followed by nested PCR (Herrewegh et al., 1995). Positive samples to FCoV RNA were subjected to a second RT-PCR assay (Addie et al., 2003). All PCR products were observed in 1,5% agarose gel.

Results

Out of 49 samples collected from dogs, 16 (32.6%) tested positive for CCoV RNA. Seven (43.8%) samples were positive for type I, and 9 (56.2%) for type II. None of the samples tested positive for both genotypes.

Out of 50 samples collected from cats, 35 (70%) were positive for FCoV RNA and were subjected to a second RT-PCR for discrimination between genotype I and II, From the 35 samples, FCoV type I was amplified in 9 (25,7%) samples, FCoV type II was detected in 6 (17%) samples and 12 (34.3%) samples showed co-infection with both types. No amplification was obtained from 8 (23%) samples.

None of the environmental samples tested positive for the presence of CCoV and 7 samples out of 8 from both cat cages showed amplification of FCoV RNA.

Discussion and conclusions

The CCoV prevalence found was consistent with previous studies. However, none of the animals was positive for both genotypes, in contrast to 76.8% of samples identified by Pratelli (2004). Eight of the positive animals also tested positive for CPV, which is in agreement with the involvement of CCoV in mixed infections. Although this finding can be due to an important environmental presence of CPV, none of these animals had clinical history of diarrhoea, supporting the idea that CCoVs aren't usually related to clinical disease in adult dogs.

Regarding FCoV, the prevalence found was higher than reported in other countries and significantly higher than previously found in stray cat population in Portugal (Duarte et al., Submitted). The large number and heavy rotation of animals in the Municipal kennel makes it difficult to implement an efficient sanitization procedure and the presence of viral nucleic acid in the environment caused by this could be responsible for this high prevalence.

Previous studies in Portugal concerning the distribution of FCoV genotypes showed a higher prevalence of FCoV type I among domestic cats (Duarte et al., 2009). Among the animals in our study we found similar prevalences for FCoV I and II and yet the percentage of co-infection within the same animal was higher than previously reported. Unfortunately we have no available data to correlate these results with the presence of the FIPV biotype.

The high prevalence of coronavirus infection found in both dogs and cats in the Lisbon Municipal Kennel allowed the viral genetic characterization, showing a high rate of co-infection with both genotypes of FCoV and absence of co-infected animals with CCoV I and II. However further investigation is needed in order to maintain a molecular epidemiological surveillance and help identify further CoV strains, as well as understand the pathogenic potential of these viruses.

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VIROLOGICAL SURVEY IN SHELTER DOGS AND CATS IN LISBON

Ricardo C ROSADO, Ana DUARTE, Augusto BAPTISTA, Filomena OLIVEIRA, Ana MACHADO, Leonel FERNANDES, Luís TAVARES

CIISA, Faculty of Veterinary Medicine, Universidade Técnica de Lisboa

Keywords: diagnostic, small animal viruses, survey

Introduction

Free-roaming stray or feral dogs and cats living in urban areas can be responsible for the spread and maintenance of several infectious diseases. These animals aren't confined or owned by anyone and both their number as well as their health status is mostly unknown to veterinarians and public health services. There have been surveys conducted in collaboration with TNR programs (trap, neuter, release), usually oriented to control cat population.

In order to conduct a virological survey of both dogs and cats 100 samples were collected from animals euthanized at the Lisbon municipal kennel. For the purpose of this study, canine distemper virus (CDV), canine parvovirus (CPV), canine coronavirus (CCoV), feline immunodeficiency virus (FIV), feline leukaemia virus (FeLV), feline coronavirus (FCoV), and feline parvovirus (FPV) were selected.

Most of these animals were captured in urban areas in and around Lisbon and a few were delivered by their owners to be euthanized for health or other issues. Since background information on the health status of these animals was lacking, antibody detection was only performed for FIV, because of its specific pathogenesis which produces persistent infection, but low level virus replication, and the fact that it has no commercial vaccine in Portugal. All other viruses were tested for the presence of antigen by ELISA or nucleic acid by polymerase chain reaction.

This information was used to estimate the prevalence of these diseases in stray and feral animals and evaluate the sanitary conditions in the kennel.

Materials and methods

Samples were collected from 50 dogs and 50 cats at the time of euthanasia, between October and November 2008 in the Lisbon municipal kennel. Biological materials included blood and faecal swabs which were kept refrigerated until processed. Blood samples were collected into tubes with EDTA, centrifuged to separate plasma from blood cells and kept at -20ºC. Faecal samples were suspended in PBS and centrifuged prior to extraction of DNA and RNA using QIAmp MiniElute Virus Spin Kit. Viral nucleic acids were kept at -80ºC until analysed.

In March 2009, 24 environmental swabs were collected from 6 occupied cells and cages randomly selected. The chosen cells were sampled every 4 days up to a total of 4 samples from each. These samples were suspended in PBS, centrifuged, and DNA/RNA was extracted following the same procedure as for the faecal swabs.

Feline plasma samples were tested for antibodies to FIV and FeLV antigen using commercial ELISA from Viracheck (Synbiotics).

Fecal swabs and environmental swabs were tested according to various protocols: For detection of Parvovirus, all samples were amplified by PCR (Desario et al., 2005). For detection of CDV and CCoV all dog samples were amplified by RT-PCR (Frisk et al., 1999, Pratelli et al., 2004). For detection of FCoV all cat samples were amplified by RT-PCR followed by nested PCR (Herrewegh et al., 1995). All PCR products were observed on 1,5%.agarose gel

Results

Table 1: Distribuition of positive results per tested samples

	dog	cat	environment
CDV	4/49		8/16
CCoV	16/49		0/24
Parvovirus	25/49	34/50	15/24
FCoV		35/50	7/8
FIV		9/50	
FELV		5/50	

Discussion and conclusions

Prevalence of CDV (8%) and CCoV (33%) were within normal range, in accordance with similar studies. Prevalences of FIV (18%) and FeLV (10%) could also be considered within normal range, although higher than previously reported in Portugal. The highest prevalences were found for FCoV (70%) and both CPV and FPV (51% and 68%).

All the analysed samples were from animals euthanized. One of the euthanasia criteria is the likelihood to be adopted, so there is a chance that results were biased towards a high prevalence of disease by choosing animals which demonstrated clinical signs of illness. Yet, from the information gathered from the animals clinical history, most were “apparently normal” on observation, and were chosen for euthanasia for their age or aggressive behaviour.

Since most of these animals were strays, one could argue that they should have come in contact with these agents during their early life and be immune to infection later on. This is especially true for viruses which provide life-long immunity like CPV and FPV. With other viruses like CDV and FCoV, the stress of capture could have induced immunosupression and reactivated latent infections. Interestingly, all animals positive to CDV RNA were also infected with CPV and three of them with CCoV, which might explain viral reactivation and subsequent excretion.

The environmental samples taken confirmed the presence of viral nucleic acid from CDV in 3 out of 4 cells, Parvovirus in 5 out of 6 cells and FCoV in both cat cages sampled during the 16 days. Even if the presence of viral nucleic acid in the environment does not equate to possibility of infection, it indicates a high number of animals present at all times hindering proper sanitization. In the case of parvovirus however, there is a strong possibility that nucleic acid found in the environment came from infectious virions, due to their extreme stability allowing them to resist adverse environmental conditions for several months.

The results found on this study, particularly on parvovirus and FCoV brought to our attention the need for a continued and more precise evaluation of the health status of free-roaming stray or feral animals in the municipal kennel, to correctly evaluate their role as viral reservoirs within and without the kennel premises,

Acknowledgements

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INTERLABORATORY VALIDATION OF REAL-TIME PCR ASSAY FOR DETECTION OF AFRICAN SWINE FEVER VIRUS

Marylène TIGNON[1], Carmina GALLARDO[2], Marie-Frédérique LE POTIER[3], Raquel NIETO[2], Elena MARTÍN[2], Virginia PELAYO[2], Evelyne HUTET[3], Yves VAN DER STEDE[4], Marisa ARIAS[2], Frank KOENEN[4]

[1]*Veterinary and Agrochemical Research Centre, Operational Directorate of Virology, Brussels, Belgium*
[2]*Centro de Investigación en Sanidad Animal-Instituto Nacional de Investigación Agraria y Alimentaria, Valdeolmos, Spain*
[3]*Agence Française de Sécurité Sanitaire des Aliments, Ploufragan, France*
[4]*Veterinary and Agrochemical Research Centre, Operational Directorate of Interaction and Surveillance, Brussels, Belgium*

Keywords: African swine fever virus, ASFV, method validation, molecular diagnosis, real-time PCR

Introduction and objectives

The agent responsible for African swine fever (ASF) is the African swine fever virus (ASFV), the only known representative of the *Asfarviridae* family. ASF is classified as a notifiable disease by the World Organisation for Animal Health (OIE). ASF clinical signs are variable. Acute forms of the disease produce congestive-haemorrhagic alterations and functional disorders in the digestive and respiratory systems. The acute and subacute forms are similar to other porcine haemorrhagic diseases, especially Classical swine fever and Erysipelas so laboratory diagnosis is essential. The epidemiological characteristics of the disease include a high rate of illness and mortality in domestic pigs and wild boars and a rapid spreading potential through direct and indirect contacts, as well as transmission through soft ticks (*Ornithodoros* spp.*)* which not only act as biological vector but also as a reservoir of the virus. The disease is generally prevalent and endemic in the majority of the countries of Sub-Saharan Africa and it has been recently introduced in Caucasus region increasing the risk of emergence in EU countries [1, 2].

Since a vaccine is currently not available, control options are limited to strict sanitary measures including culling of infected and potentially infected animals and vector elimination. A rapid and sensitive diagnosis of the disease is a key factor to reduce the spread of the disease and thereby limiting major economic losses due to ASF. In this context, a new real-time polymerase chain reaction (rtPCR) assay has been developed for the molecular diagnosis of ASF. The assay is multiplexed for simultaneous detection of swine beta-actin as an endogen internal control.

Materials and methods

Control plasmid

The gene P72 from ASFV Lisbon60 strain was amplified by PCR and cloned into pCR2.1 vector (Invitrogen). Recombinant plasmid was linearised by restriction and quantified by spectrophotometry.

Virus reference panel

Forty-one ASFV strains representative of genotype I, II, V, VIII, IX and X, with different historical and geographical origins, available in the ASF collection held at CISA-INIA, the EU reference laboratory for ASF, were included in this study.

Samples from experimentally infected animals

One hundred eleven serum samples were collected from domestic pigs infected with ASFV strains from genotype I, IX or X at different times post infection (p.i.). Virulence of the strains ranged from acute (I, IX) to sub-acute (X).

DNA extraction

NucleoSpin (Macherey-Nagel) and QIAamp DNA (Qiagen) kits were used for DNA extraction from stock viruses and serum samples according to manufacturer's instructions. Qiaprep spin miniprep kit (Qiagen) was used for plasmid extraction.

Primers and probe

ASFV-specific primers and probe were designed upon the consensus sequence obtained from more than 180 publicly available ASFV P72 gene sequences. The p72F (5'-TGCTCATGGTATCAATCTTATCG-3') and p72R (5'-CCACTGGGTTGGT ATTCCTC-3') primers amplified a 159 bp region targeted by the ASF-p72-probe (5'-FAM-TTCCATCAAAGTTCT GCAGCTCTT-TAMRA-3'). Specific primers and probe targeting swine beta-actin gene were included in the assay as endogen control [3].

Procedure of the rtPCR assay

The rtPCR was carried out using the QuantiTect Multiplex PCR kit (Qiagen) with 0.6µM of each ASF-specific and swine actin primers, 0.3µM ASF-p72-probe and 0.2 µM sw-actin TET-BHQ1 labelled probe. Aliquot of 5 µl DNA was added to 20 µl of PCR mix.

The cycling protocol was run as follows: 50°C for 2 min., 95°C for 10 min. and 45 cycles with 95°C for 1 min. and 60°C for 1 min. Assays were performed on three different real-time PCR thermal cyclers (7500 FAST from Applied Biosystems, Mx3005P from Stratagene and Chromo4 from Biorad). Fluorescence values were collected during the annealing step and analysed with the appropriate software.

The presence of the ASFV genome was also confirmed by conventional PCR assay [4] and rtPCR assay [5].

Validation of the new rtPCT assay

A tenfold serial dilutions from $5.7x10^{4}$ to $5.7x10^{-5}$ copies/5 µl of control plasmid were analysed by rtPCR assay, including three replications per assay and three assays per laboratory.

Statistical analysis

Repeatability and reproducibility were determined using variance analysis in SAS statistical software and expressed as coefficient of variation (CV).

Results

Linearity, limit of detection and precision

The linearity of the rtPCR assay was demonstrated using titration of recombinant plasmidic DNA in the range of 57 to $5.7x10^{4}$ copies/5 µl. In this range, the efficacy was evaluated between 96 and 121 %. The limit of detection was estimated between 5.7 and 57 copies per reaction. At the intra-laboratory level CV remained below 3.3% for repeatability and 3.6% for intermediate precision. Inter-laboratory reproducibility CV was 6.6%.

Intra-species specificity

The specificity of the assay was demonstrated by the detection of all the tested strains, including recent circulating isolates originated from Sardinia (Italy), Caucasus and East Africa.

Diagnostic evaluation on samples from experimentally infected animals

Compared to the conventional PCR [4] and existing rtPCR [5] assays, the viral genome was generally detected at an earlier stage post infection (from 3 to 7 days p.i) in serum samples. The virus could also be detected at a later stage of infection (from 42 days and up to 68 days p.i.) by the new assay. The positive deviation was evaluated to 25.6 and 38.4% compared to conventional PCR and rtPCR respectively and the negative deviation to 8%.

Discussion and conclusions

The new rtPCR assay has been validated by the European and two national reference laboratories whereby repeatability and reproducibility have been demonstrated. The assay presents a high sensitivity and specificity, including for the currently circulating isolates. Moreover, the strong positive deviation observed on samples from experimentally infected animals highlighted the potential of the new rtPCR assay. It is particularly interesting for detection of acute and subacute virulent strains at early and late infection stages.

The new rtPCR assay will provide a rapid, sensitive and reliable molecular diagnostic tool for ASF diagnosis that can be useful both for surveillance in ASF-free areas and control in endemic areas.

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AVIAN NEPHRITIS VIRUS COMMONLY DETECTED BY RT-PCR IN FIELD AND LONGITUDINAL SAMPLES FROM BROILER FLOCKS

Daniel TODD[1], Nigel MCBRIDE[1], James TRUDGETT[1], Brendan DONNELLY[1], Francis MCNEILLY[1], Victoria J. SMYTH[2], Heather L. JEWHURST[2], Brian M. ADAIR[1]

[1]Agri-Food and Biosciences Institute, Stormont, Belfast , BT4 3SD, Northern Ireland;
[2]Queen's University, Agri-Food and Biosciences Institute, Stormont, Belfast , BT4 3SD, Northern Ireland

Keywords: ANV, astrovirus, detection, RT-PCR

Introduction and objectives

The astrovirus avian nephritis virus (ANV) was first identified in Japan in 1979. Experimental infection of day-old chicks resulted in diarrhoea, growth retardation, mortality and interstitial nephritis. Serological studies have shown that ANV infections are common in commercial poultry and probably occur worldwide. The detection of ANVs in broiler flocks with severe growth problems including runting-stunting syndrome and the ability of ANVs to cause substantial degrees of growth retardation following experimental infection of SPF and broiler chickens suggest that ANV infections contribute to broiler growth problems in the field. However, their clinical significance remains largely unknown due to the lack of convenient diagnostic tests. Knowledge of the ANV genomic sequence has allowed the development of RT-PCR tests. Using primers based in the protease (ORF1a) gene, Mandoki et al. [2] confirmed the presence of ANV in 36/56 kidney samples from clinical outbreaks of nephritis and gout in Hungary, where diarrhoea and growth retardation were also observed. An RT-PCR test based on the use of degenerate primers sited in the polymerase (ORF1b) gene was described in a US study by Day et al. [1], who reported the detection of ANVs in enteric samples from sick and healthy chickens and turkeys originating in the US. The detections of ANV RNA by these RT-PCRs may have been limited by both the high sequence diversity of ANV and the relatively low availability of sequence data. We have recently determined partial nucleotide sequences of over 16 ANVs, and have shown that the 3` untranslated region (3`UTR) of the ANV genome displays conserved sequences on which to base the design of RT-PCR primers. In this paper we report the development of a sensitive RT-PCR test for detecting ANV RNA and its application to samples from broiler flocks with growth problems, and to longitudinal survey samples of 4 broiler flocks.

Materials and methods

Samples of faeces or gut contents were obtained from sick chickens collected from UK and German broiler flocks, that were experiencing growth depression problems between 2004 and 2008. In longitudinal surveys of 4 broiler flocks that displayed below average performances, 12 birds were sampled at days 0, 4 or 5, 7, 14, 21 and 28, and each set of 12 were processed by homogenisation to produce 4 gut content and 4 kidney pools. RNAs were extracted from homogenates and from the avian astrovirus isolates using QIAamp viral RNA or RNeasy extraction kits (Qiagen), and subjected to one step RT-PCR using forward and reverse primers, located within the 3`UTR of the ANV genome, which flanked an amplicon of 182bp. Reverse transcription (45°C for 30 min) and initial denaturation step (94°C for 2min) was followed by 40 PCR cycles, each comprising denaturation (94°C for 30sec), annealing (50°C for 30sec), and extension (68°C for 30sec). PCR products were analysed by agarose gel electrophoresis and visualised following ethidium bromide staining using UV transillumination. Amplicons obtained with 14 field samples were sequenced to demonstrate ANV- specificity. The test was also applied to RNAs extracted from the 11672, FP3 and 612 isolates of chicken astrovirus (CAstV), and duck hepatitis viruses types 2 (DHV2) and 3 (DHV-3) to investigate specificity. The sensitivity of the test was estimated from a 10-fold dilution series of *in vitro* transcribed RNA of known concentration from a cloned 394 bp ANV amplicon.

Results

Sensitivity and specificity. The limit of detection (LOD) of the RT-PCR assay was determined to be between 18 and 180 viral copies. This was done using 10-fold serial dilutions (in water) of RNA that had been *in vitro* transcribed from a recombinant plasmid containing a 394bp fragment encompassing the 3`UTR of ANV. RT-PCR products were not amplified with RNAs that had been extracted from samples of DHV-2, DHV-3 and the 11672, FP3 and 612 isolates of CAstV, indicating that the RT-PCR test was specific for ANV and not other avian astroviruses.

Application of RT-PCR tests to field and longitudinal samples. Gut content or faeces samples that had been collected from broiler chicken flocks experiencing severe growth retardation problems were tested by the ANV RT-PCR test. All 55 (100%) samples tested positive by RT-PCR. With the majority of field samples a single DNA band, sized 182 bp, was detected after agarose gel electrophoresis, ethidium bromide staining and UV transillumination. In a small (<5%) number of samples additional DNA bands, with inconsistent sizes, were detected in addition to the 182 bp amplicon. When the RNAs from 12 representative field samples were tested using previously described RT-PCR tests [1,2], 9 were found to be positive with by both tests. 80/80 pooled gut content and 79/80 pooled kidney samples from the longitudinal survey samples collected from 4 broiler flocks on days 4 or 5, 7, 14, 21 and 28 were positive by the ANV RT-PCR test. However, none of the 16 gut content or 16 kidney samples collected on day 0 were positive.

Sequence characterisation of RT-PCR amplicons. Nucleotide sequences, approximately 110 nt in size, were determined for 14 amplicons generated with field samples. These were shown to be ANV-specific, sharing between 83% and 97% nucleotide identity with the G4260 serotype 1 ANV strain.

Discussion

Here we describe a sensitive, specific RT-PCR test for detecting ANV RNA. The test is based on primer sequences located within the 3`UTR, which we have shown displays relatively high levels of sequence conservation when compared to other genomic regions of ANV (Todd, upublished results). A small-scale comparison showed that two previously described RT-PCR tests failed to detect ANV in 3 of 12 field samples that were found to be posititive using our test, suggesting that the primers used in the previously described tests were mis-matched with some ANV sequences. The detection rates obtained with the 55 (100%) field samples (gut content or faeces) and with 159/192 (83%) longitudinal survey samples (gut content and kidney) at timepoints from day 0 to day 28 confirmed the high sensitivity of the test and demonstrated the high prevalence of ANV infections in broiler flocks with growth problems and below average performances. The absence of positive RT-PCR signals in the day 0 samples and their presence in day 4 samples showed that detectable infections occurred very soon after the introduction of the chicks into the broiler house. The detections of ANV in the gut and kidney at all timepoints after day 4 suggested that ANV infections are not rapidly cleared. However, the possiblity that the flocks were successively infected with different ANV types cannot be ruled out. Athough lower detection rates may be obtained with broiler flocks displaying above average performances, the high sensitivity of the test and the apparent high prevalence of ANV infections suggests that RT-PCR may be of questionable value as a disease diagnostic given that many subclinical infections may be detected. It is possible that the virus load may prove to be a useful indicator of disease and that a quantitative real time RT-PCR may be more valuable for assessing the clinical importance of ANV infections in broilers.

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SEROPREVALENCE OF CHICKEN ASTROVIRUS INFECTIONS

Daniel TODD[1], Donna L. WILKINSON[2], Mildred WYLIE[1], Heather L. JEWHURST[2], Brian M. ADAIR[1]

[1]Agri-Food and Biosciences Institute, Stormont, Belfast , BT4 3SD, Northern Ireland;
[2]Queen's University, Agri-Food and Biosciences Institute, Stormont, Belfast , BT4 3SD, Northern Ireland

Keywords: astrovirus, chicken, seroprevalence

Introduction and objectives

A novel avian astrovirus, named chicken astrovirus (CAstV), was first characterised in 2004 on the basis of partial nucleotide sequencing [1]. Sequence-related CAstVs have since been detected in the USA [3]. We have recently characterised antigenically different CAstVs, originally called enterovirus-like viruses (ELVs), and described their phylogenetic relationship to other avian astroviruses [5]. Our interest in CAstVs results from their detection in broiler flocks experiencing growth retardation problems including runting-stunting syndrome and from experimental infections of day-old SPF chickens, in which variable degrees of growth retardation were obtained [2]. The 11672 and 612 isolates of CAstV represent 2 different antigenic types, which share low levels of cross-reactivity by indirect immuno-fluorescence (IIF) [5]. CAstV11672 is very closely related antigenically to the FP3 isolate of CAstV, which was isolated as an ELV from dead-in-shell embryos in the UK in 1984 [4], whereas CAstV612 is more closely related to the first described CAstV isolate (refCAstV) [1]. So far, it has not been possible to grow the 11672 and 612 isolates productively in cell culture, and this has hindered their serotypic characterisation and the development of a serum neutralisation (SN) test with which type-specific anibodies could be detected. However, the partial growth of both CAstVs in primary chick embryo cell lines have allowed separate IIF tests to be developed for detecting antibodies to each CAstV. The objective of this work was to determine the seroprevalences of CAstV infections in four generations of chickens involved in commercial production.

Materials and methods

Indirect immunofluorescence (IIF) tests were performed using CAstV-infected cells grown on circular glass coverslips [3]. Coverslip cultures were incubated for 1h at 37°C with dilutions (1 :100 used for screening) of chicken serum samples. After washing bound chicken antibody was reacted with FITC-conjugated goat anti-chicken Ig (usually 1:100) for 1h at 37°C, with immunofluorescent staining being detected using a UV microscope. Experimental antisera to CAstV11672 and CAstV612 were prepared as described [3]. Most serum samples were obtained from 3 UK poultry organisations (A, B and C) during 2005-07, with broiler, parent, grandparent (GP) and great grandparent (GGP) flocks being represented. Pooled serum samples prepared by generating 3 pools (each comprising 4 sera) from 12 individual serum samples were obtained from additional UK and European parent flocks. Ninety nine turkey serum samples collected from 10 breeder flocks (aged 30-50 weeks) were obtained from organisation B.

Results

Reaction of CAstV11672-infected CEL cells and CAstV612-infected CEK cells with experimental or field sera produced intracytoplasmic immunofluorescent staining, with some cells showing brightly-staining, granular inclusions and other cells showing more evenly dispersed staining patterns. With both the CAstV612 and CAstV11672 IIF tests a serum dilution of 1:100 was selected for flock testing purposes on the basis that lower dilutions eg 1:20 dilutions produced high levels of non-specific staining.

All 10 broiler flocks (aged 40 days) from organisation A were antibody-positive for both CAstVs viruses, with CAstV612 seropositivities ranging from 50 to 95% (mean 73%) and CAstV11672 seropositivities ranging from 26 to 64% (mean 46%). When samples (n=7-30) from parent flocks (23-26 wks) from organisations A, B and C were tested, 23 of 24 and 27 of 28 flocks were positive for antibody to CAstV612 and CAstV11672 respectively. CAstV612 seropositivity ranged from 0 to 90%, and CAstV11672 seropositivity ranged from 0 to 77%. When 4 parent flocks were re-tested at 46 weeks, 3 flocks showed similar or reduced seropositivity to both CAstVs, whereas the seropositivities of 1 flock increased significantly ($p=< 0.05$) to both CAstV11672 and CAstV612. Taking the serum samples from broiler parent flocks as a whole, 348 (45%) of 772 samples were positive for CAstV612 antibody and 304 (35%) of 880 samples were positive for CAstV11672 antibody.

Testing of serum samples (n = 9-30) from GP flocks (20-26 wks) from organisation A and C showed that 14 of 18 (78%) and 5 of 18 (28%) were positive for CAstV612 and CAstV11672 antibody respectively, with mean seropositivities estimated as 26% (CAstV612) and 12% (CAstV11672). When tested (n =~10) at 23-30 wks, 2 of 5 GGP flocks from organisation C were positive for CAstV612 antibody (range 0 to 50%; mean 12%) whereas none of the 5 flocks were seropositive to CAstV11672. Increased CAstV seropositivity levels were observed when some GP and GGP flocks were re-tested at 48-57 weeks.

Using a screening test, in which 12 serum samples were tested as 3 separate pools, 21 (58%) and 23 (64%) of the 36 UK broiler parent flocks were seropositive for CAstV612 and CAstV11672 respectively. Of the 10 UK organisations surveyed, flocks from 9 (90%) organisations were positive for CAstV612 antibody and flocks from 8 (80%) organisations were positive for CAstV11672 antibody. Parent flocks from 8 European countries including Croatia, the Czech Republic, Denmark, Germany, Ireland, the Netherlands, Norway and Sweden were seropositive for both CAstV612 and CAstV11672. Of 99 samples obtained from 10 turkey breeder flocks (20-50 weeks), 9 (9%) derived from 2 flocks were positive for CAstV612 antibody and 2 (2%) samples from a single, different flock were positive for CAstV11672 antibody.

Discussion

The high seropositivities (at~40 day-old) indicate that infections with both CAstV types are common in broiler flocks, and that most birds are infected within 1 or 2 weeks. It is likely that most broilers will be infected with horizontally-acquired virus. Contamination within the house is likely to be high, given that astroviruses are excreted in large numbers and show high resistance to inactivation. Although CAstV infections are also prevalent (>95%) in broiler parent flocks (20-26 wks), in-flock seropositivity values suggest that progeny chicks will have variable levels of maternally derived antibody (MDA). Chicks without MDA are more likely to be susceptible to more severe pathogenic effects, which may arise if CAstV spreads to internal organs such as the kidneys. Re-testing of parent, GP and GGP flocks during lay indicated that in-lay infections occur, and this provides the opportunity for vertical transmission via the egg from infected parent to progeny chick. Not unexpectedly, CAstV infections were less prevalent in GP and GGP flocks, which operate with much greater biosecurity. On the basis of these detections and the likelihood that CAstVs can be vertically transmitted, SPF flocks supplying eggs for vaccine production should be screened for CAstV infections.

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VALIDATION OF A REAL-TIME RT-PCR FOR THE DETECTION OF BLUETONGUE VIRUS IN BULL SEMEN AND DEVELOPMENT OF AN INTERNAL CONTROL

Tine VANBINST, Frank VANDENBUSSCHE, Kris DE CLERCQ

Veterinary and Agrochemical Research Centre, Department of Virology, Brussels, Belgium

Keywords: bluetongue virus, bull semen, internal control, real-time RT-PCR, validation

Introduction and objectives

Bluetongue (BT) is an arboviral disease of domestic and wild ruminants which constitutes an important impediment for international trade. The presence of bluetongue virus (BTV) in semen has been demonstrated intermittently in naturally infected bulls and rams. For BTV-free countries BTV transmission via artificial insemination forms a major threat and importation of frozen semen samples are under strict control. Certificates of frozen semen for importation are only provided after a combination of precautionary measures that are taken and diagnostic tests on the donor's blood samples that are performed. These diagnostic tests, described by the OIE, are performed on multiple time-points and may include antibody detection or antigen identification using virus isolation or RT-PCR. Analysis of blood instead of semen does confer a great disadvantage for international trade as many semen samples from a BTV-infected bull may be free of BTV, even during the vireamic phase. The time-lag before release of semen samples due to the period between examinations is another drawback. Here we describe the validation of a duplex real-time RT-PCR assay, including RNA extraction, to analyze frozen semen samples for the presence of BTV RNA. An internal control assay was developed to monitor the RNA quality and/or accurate RNA extraction of bull semen.

Materials and methods

Two different RNA extraction methods were tested on artificially BTV-8 positive semen samples: the commercially available NucleoSpin RNA Virus kit (Machery-Nagel) and a Trizol-based RNA extraction method previously optimized for processing *Culicoides* (2). The artificial samples were prepared by spiking frozen semen from seronegative bulls with a five-fold dilution series of a Belgian BTV-8 field isolate (Bel2006/01). Control samples were also prepared by spiking cell culture medium. The ratio of virus solution to semen or cell culture medium was 1 to 10.

Positive frozen semen samples from donor bulls, naturally infected with BTV-8 during the 2006 outbreak in Belgium, were obtained from an artificial insemination centre. All semen samples were divided into 10 straws (= 1 batch). The negative frozen semen samples used in this study were collected by two artificial insemination centers before the onset of the first BT-8 epidemic in Belgium.

All duplex real-time RT-PCR analyses were carried out using the RNA-UltraSense One-Step qRT-PCR kit (Invitrogen). The BTV group-specific primers and Taqman probe targeting BTV-segment 5 are cited in Toussaint *et al.* (1). A novel pair of primers and TaqMan probe for internal control, GAPDH-mRNA, was designed based on the bovine GAPDH genome (GeneBank accession nr: NC007303) using Visual OMP Software (DNA Software Inc.)

Results

A duplex real-time RT-PCR was optimized which amplifies the BTV- and GAPDH-targets simultaneously. To identify the most suitable procedure for RNA extraction from frozen semen samples, total RNA was extracted from BTV-8 spiked semen and control samples using the NucleoSpin RNA Virus kit and an in-house Trizol-based method (2). The highest quantity of amplified RNA, expressed as Cycle threshold (Ct), in the semen samples was observed in the ones processed with the Trizol-based method (Figure 1). The curves of the BTV-8 dilutions from the semen and control samples almost overlapped (no matrix effect). The analytical sensitivity of the RT-qPCR was evaluated by comparing the real-time RT-PCR assay with virus isolation on serial dilutions of two positive semen samples from the field. Virus isolation was performed by intravenous inoculation of embryonated hens' eggs. In both cases, real-time RT-PCR was more sensitive than virus isolation. The analytical sensitivity of the assay or the detection limit has yet to be determined. To evaluate the sensitivity and the specificity, the real-time RT-PCR assay was conducted on positive semen samples (n = 89) from BTV-8 naturally infected bulls (n = 19) and negative semen samples from uninfected bulls (n = 48). Seventy-eight samples from the infected bulls tested positive for BTV. Concerning the remaining semen batches (n = 11) that were BTV-negative, we checked 5 additional straws of each batch (total n = 55). Surprisingly, nine of these batches were actually positive (n = 22), but they all contained a small viral load (Ct = 34.8 – 40.0). In three of them only one in five straws was positive. The real-time RT-PCR assay generated a negative result with all negative samples tested. The average result of internal control GAPDH for all field samples, 27.3 Ct (95% CI: 27.14 – 27.46), demonstrated that the batches were of excellent quality.

Discussion and conclusions

In the present study, we developed a specific, sensitive and rapid RNA extraction method and duplex real-time RT-PCR assay for the detection of BTV and an internal control in frozen semen samples. Analysis of semen samples from the field demonstrated that bulls with naturally acquired BTV-8 infection shed virus in their semen. The varying results of different straws from the same batch show that BTV was not consistently divided. Although additional research is certainly necessary, our preliminary results indicate that analysis of at least five straws per batch are recommended. Diagnostic tests for importation of frozen semen that are directly performed on the semen samples confer some major advantages: multiple analyses are no longer necessary and consequently time and costs are reduced. Moreover, it prevents the loss of a lot of semen samples due to 'false-positive' results. The protocol described here is a valid test for analyzing semen batches individually for the presence of BTV viral RNA, and may be of great help to international trade of bull semen.

Acknowledgements

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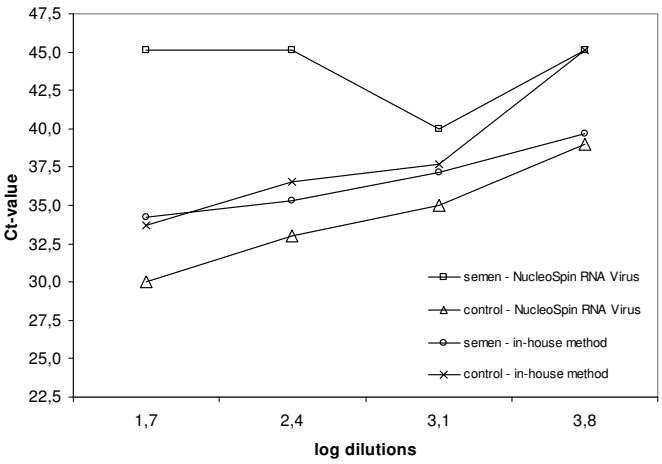

Fig 1. Comparison of real-time RT-PCR results of RNA extracted from semen and control samples spiked with a dilution series of BTV-8 (5-fold) using two different extraction methods: NucleoSpin RNA Virus kit and an in-house Trizol-based method.

DESCRIPTION AND VALIDATION OF FOUR REAL-TIME RT-PCR FOR THE SEROTYPING OF BTV-1, BTV-6, BTV-8 AND BTV-11

Frank VANDENBUSSCHE, Ilse DE LEEUW, Kris DE CLERCQ

Veterinary and Agrochemical Research Centre, Department of Virology, Section of Epizootic Diseases, Brussels, Belgium

Keywords: BTV-1, BTV-6, BTV-8, BTV-11, real-time RT- PCR

Introduction and Objectives

Bluetongue (BT) is an arthropod-borne, non-contagious viral disease of domestic and wild ruminants that induces variable clinical signs depending on the host species and the breed. Until recently, bluetongue virus (BTV) was mainly confined to tropical and temperate areas, but since 1998 BTV strains belonging to 5 serotypes (BTV-1, BTV-2, BTV-4, BTV-9 and BTV-16) have been circulating across large parts of the Mediterranean Basin. In August 2006, BTV emerged for the first time in Central-Western Europe and quickly became disseminated over the Netherlands, Belgium, Germany, Luxembourg, Switzerland, the half of France, and the South-East of the United Kingdom. Virus isolation and subsequent serological/molecular characterization demonstrated that the epizootic was caused by a BTV-8 strain, which had previously not yet been detected in Europe (1). In 2007 a BTV-1 strain was introduced from Morocco into South of Spain. Due to animal transport the virus subsequently spread to the North of Spain and large parts of France (2). In 2008, a BTV-6 strain was identified in the Netherlands and Germany (3). In the same year, a BTV-11 strain was detected in a small number of animals in Northern Belgium (4).

Currently, 4 BTV serotypes are thus present in Central-Western Europe which greatly complicates the control policy. Therefore, the main goal of the present study was to develop 4 genotype specific real-time RT-PCRs (RT-qPCR) for the detection of the BTV-1, BTV-6, BTV-8 and BTV-11 strains.

Materials and methods

The genotype specific RT-qPCR assays were designed on segment 2 which encodes the main serotype-determining outer capsid protein of BTV. All available sequences of segment 2 were obtained from the GenBank database and aligned using the Clustal W software. BTV-1, BTV-6, BTV-8 and BTV-11 specific primers and Taqman probes were designed using Visual OMP software (DNA software). Several locked nucleic acid residues (LNAs) were incorporated in the Taqman probes to increase their binding affinity. All RT-qPCR reactions were performed with the RNA-Ultrasense One-Step qRT-PCR System (Invitrogen) according to the manufacturer's instructions and run on a LightCycler 480 instrument (Roche diagnostics). RT-qPCR assays were optimised by adjusting the primer and probe concentrations according to standard procedures.

After initial optimisation, the RT-qPCR assays were partially validated. The analytical sensitivity of the assays was determined by testing a 2-fold dilution series of BTV-1, BTV-6, BTV-8 and BTV-11 cell culture supernatant in BTV-negative blood. The analytical specificity was assessed by analysing the 24 BTV serotypes from the European Union Reference Laboratory and 200 blood samples which were negative in the pan-BTV specific RT-qPCR (5). Blood samples spiked with different concentrations of BTV-1, BTV-6 and BTV-8 were used to compare our in-house assays with 3 commercially available BTV-1, BTV-6 and BTV-8 assays from the 'Laboratoire Service International (LSI)' (i.e. triplex FCO BTV1 genotyping, FCO BTV6 and triplex FCO BTV8 genotyping). There were no commercial kits available for the detection of BTV-11.

Results

The analytical sensitivity of the assays was determined by repeated testing a 2-fold dilution series of BTV-1, BTV-6, BTV-8 and BTV-11 in BTV-negative blood. Probit analysis indicated that the limit of detection of the genotype specific and pan-BTV specific RT-qPCR assays are comparable. The analytical specificity of the assays was assessed by (i) testing the 24 reference strains of all BTV serotypes and (ii) 200 BTV-negative blood samples. Positive test results were only obtained for the 4 reference strains to which the assays were designed. No false-positive test results were observed with any of the other reference strains or the BTV-negative blood samples. The linear range and PCR-efficiency of the assays were determined by analysing a 10-fold dilution series of BTV-1, BTV-6, BTV-8 and BTV-11 in PBS. All assays exhibited a linear range of at least 10^{-2} to 10^{5} $TCID_{50}$/ml with an R^2 > 0.99 and a PCR efficiency >93% (Fig 1). A comparative analysis between our in-house assays and 3 commercially available BTV-1, BTV-6 and BTV-8 RT-qPCR assays was performed by testing 96 spiked blood samples in both assays. Bland-Altman and Passing-Bablok regression analysis indicated that the mean difference between the in-house and commercial assays is negligible and that most results fell within the 95% confidence interval of the mean. Both assays (i.e. in-house versus commercial) can therefore be used interchangeably.

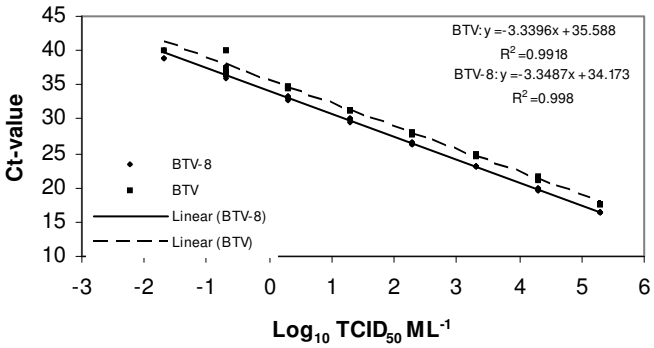

Fig. 1. Detection of BTV-8 dilutions with the BTV-8 specific RT-qPCR and the Pan RT-qPCR. Each dilution series was tested in 5-fold.

Discussion and conclusions

The simultaneous presence in Central-Western Europe of BTV-1, BTV-6, BTV-8 and BTV-11 greatly hampers the control policy of this disease. When more than one serotype co-exists in the same area, the use of a pan-BTV specific assay is no longer sufficient as it does not allow differentiating among the various serotypes. In Belgium, the identification of a new BTV serotype such as BTV-1, BTV-6 or BTV-11 has totally different consequences than the identification of the endemic BTV-8 serotype. As the current BTV-8 vaccination campaign will not protect ruminants against other serotypes, the 3 recently introduced serotypes could spread throughout the rest of the country as soon as the weather conditions are favourable for vector activity. Rapid, sensitive and specific assays are therefore needed to correctly identify the circulating BTV strains in field samples. As soon as the serotype of the circulating BTV strain is determined, the most appropriate control measures can be taken to minimize the spread of the virus. Transport can be banned in a certain area, infected animals can be slaughtered and a vaccination campaign can be started. The genotype specific assays described in this study will therefore greatly facilitate the control policy and hopefully help to stop the dissemination of the recently introduced serotypes.

Acknowledgements

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2. Promed 20081029.3412

PROXIMITY LIGATION ASSAY (PLA) IS A NEW METHOD FOR THE DETECTION OF AVIAN VIRUSES

Alia YACOUB[1], Joerg SCHLINGEMANN[1,3], Mikael LEIJON[1], István KISS[1], Siamak ZOHARI[1], Anna MATYI-TOTH[1], Ann NORDENGRAHN[4], Ulf LANDEGREN[2,3], Bjorn EKSTROM[2] & Sándor BELÁK[1]

[1]Department of Virology, Immunobiology and Parasitology, National Veterinary Institute (SVA), SE-751 89 Uppsala, Sweden;
[2]Olink AB, Uppsala Science Park, Dag Hammarskjölds väg 54A, 75183 Uppsala, Sweden
[3]Rudbeck Laboratory, Department of Genetics and Pathology, Uppsala University, Dag Hammarskjölds väg 20, SE-75685 Uppsala, Sweden;
[4]Svanova Biotech AB, Uppsala Science Park, Dag Hammarskjölds väg 32A, 75183 Uppsala, Sweden

Keywords: avian viruses, PLA

Introduction and objectives
The highly pathogenic avian influenza virus threatens to become a worldwide pandemic (Mansfield, 2007;) Avian influenza viruses (AIV) belong to type A, which is able to infect a range of species including humans, birds, horses and pigs (Webster et al., 1992). On the basis of the antigenicity of haemagglutinin (HA) and neuraminidase (NA) glycoproteins, influenza A viruses are classified into 16HA and 9NA subtypes. Virus isolation (VI) in embryonated chicken eggs followed by subtype determination is still considered the golden standard method for AIV characterization but is slow and laborious. New detection methods and fast screening are becoming increasingly important.

The objective of this work was to develop a new diagnostic method for the detection of avian viruses with high accuracy and good sensitivity.

Materials and methods
A novel proximity ligation assay (PLA) using specific monoclonal antibodies (Mab) was developed for the detection of avian influenza viruses. The technique is based on detection of proteins of a microorganism in biological samples. In PLA, affinity probes that bind target proteins are equipped with DNA strands that can be joined by ligation when two or more such reagents are brought into proximity by binding to the same target molecule. The DNA ligation products are subsequently detected by PCR amplification using fluorogenic probes. Infected allantoic fluids by different avian influenza subtyes were used in this assay (H1-H7, H9-H12). There are preliminary results for the detection of other avian viruses like Newcastle disease virus (NDV) and Infectious laryngotracheitis virus (ILTV) by PLA.

Results
All AIV subtypes tested are readily detected by the proximity ligation assay with signals between five and six orders of magnitude above the background signal. Other heterologous avian viruses showed a signal almost below or near the level of the background, an indication of the specificity of the assay. The sensitivity of the assay was calculated and showed that PLA assay is more sensitive than antigen capture ELISA and comparable to the golden standarard real-time RT-PCR for Influenza matrix gene detection.

Discussion and conclusions
We present here a novel method for the protein analysis where antibody-based detection of a target protein via a DNA ligation reaction results in a formation of an amplifiable DNA strand that can be analyzed by real-time PCR. Our results are comparable to real-time PCR assay (Spackman et al., 2002).

In conclusion, PLA is a simple and reliable method for the detection of viral infection and the system needs no sample preparation and RNA extraction. The system can be used for analysis of hundreds of samples. Multiplexed proximity ligation assay can be developed for different infectious agents simultaneously.

Acknowledgements
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INVESTIGATION OF AVIAN INFLUNZA VIRUS (AIV) IN WILD BIRDS IN TURKEY

Harun ALBAYRAK, Emre OZAN

Veterinary Control and Research Institute, Virology Laboratory, Samsun, Turkey

Keywords: avian influenza virus, real-time RT-PCR, Turkey, wild bird

Introduction and objectives
Avian influenza (AI) is caused by specified viruses that are members of the family Orthomyxoviridae and placed in the genus influenzavirus A. There are three influenza genera – A, B and C; only influenza A viruses are known to infect birds. Diagnosis is by isolation or detection and characterisation of the virus. Influenza A viruses have antigenically related nucleocapsid and matrix proteins, but are classified into subtypes on the basis of their haemagglutinin (H) and neuraminidase (N) antigens (80). At present, 16 H subtypes (H1–H16) and 9 N subtypes (N1–N9) are recognised. To date, the highly virulent influenza A viruses that produce acute clinical disease in chickens, turkeys and other birds of economic importance have been associated only with the H5 and H7 subtypes. Most viruses of H5 and H7 subtype isolated from birds, have been of low virulence for poultry. Due to the risk of a H5 or H7 virus of low virulence becoming virulent by mutation, all H5 and H7 viruses have been identified as notifiable avian influenza (NAI) viruses. The aim of this study was to survey organ pools obtained from a variety of wild avian species in Kizilirmak delta and the adjacent wetlands of Turkey for the presence of Avian Influenza.

Materials and methods
A total of 60 wild birds surveyed in this study were sampled for the presence of Avian Influenza virus (AIV) by Taqman-based real-time reverse tarnscriptase polymerase chain reaction assay (rRT-PCR) and reverse-transcriptase polymerase chain reaction assay (RT-PCR).

Results
Samples were tested by Taqman-based realtime RT-PCR and RT-PCR for the presence of Avian Influenza (AIV). No viral nucleic acid was detected from all samples by RT-PCR (matrix protein primers), but one viral nucleic acid was detected from Buzzard (*Buteo buteo)* by rRT-PCR (matrix protein primers and prob). This viral nucleic acid was not determined by rRT-PCR (H5 and H7 primers and probes).

Discussion and conclusions
Although many avian influenza viruses were isolated from domestic poultry in northern of Turkey, only one avian influenza viral nucleic acid was detected from wild birds in the same region, in this study. This viral nucleic acid is neither H5 nor H7. Additionally, viruses isolated from this region are H5N1. The reason of this is not clear for us.

Acknowledgements
We are greatful to Nicholas Johnson (Veterinary Laboratories Agency, Weybridge, UK) for control RNA, cDNA and his helpful comments.

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FIRST INVESTIGATION OF WEST NILE VIRUS (WNV) IN WILD BIRDS IN TURKEY

Harun ALBAYRAK, Emre OZAN

Veterinary Control and Research Institute, Virology Laboratory, Samsun, Turkey

Keywords: Real-time RT-PCR, turkey, West Nile virus, wild bird

Introduction and objectives
West Nile (WN) virus is an arthropod-borne virus that is taxonomically classified with in the family *Flaviviridae*, genus *Flavivirus*, and that is a member of the Japanese encephalitis (JE) virus serocomplex, which includes JE virus, St. Louis encephalitis (SLE) virus, and Murray Valley encephalitis virus. WNV has a wide geographical range that includes portions of Europe, Asia, Africa, Australia (Kunjin virus) and in North, Central and South America. The aim of this study was to survey organ pools obtained from a variety of wild avian species in Kizilirmak delta and the adjacent wetlands of Turkey for the presence of WNV.

Materials and methods
A total of 60 wild birds surveyed in this study were sampled for the presence of West Nile virus (WNV) by Taqman-based real-time reverse tarnscriptase polymerase chain reaction assay (rRT-PCR).

Results
Samples were tested by Taqman-based realtime RT-PCR for the presence of west nil virus (WNV), but no viral nucleic acid was detected from all samples.

Discussion and conclusions
This datum was emerged that wild birds do not infected with West Nile Virus (WNV) in Northern of Turkey.

Acknowledgements
We are greatful to Nicholas Johnson (Veterinary Laboratories Agency, Weybridge, UK) for control RNA, cDNA and his helpful comments.

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LONG-TERM MONITORING OF WILD BIRDS FOR ORTHO- AND PARAMYXOVIRUSES IN NORTH-WESTERN ITALY

Rosalia CENTORBI[1], Francesca RIZZO[1], Riccardo ORUSA[2], Elisa BARCUCCI[1], Maria Lucia MANDOLA[1]

[1]*Lab. Spec. Diag. Molec. Virol. e Ovocoltura Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (IZS PLV), sede di Torino, Italy;*

[2]*Centro di Referenza Nazionale per le malattie degli Animali Selvatici (Ce.R.M.A.S), Aosta, Italy*

Keywords: avian influenza, paramyxovirus, surveillance, wild birds

Introduction and Objectives

The avian heritage of Piedmont includes about 18 milions raised heads distribuited in more than 2400 installations. About 900 of them are monitored for avian influenza (data 2008) through mandatory investigation, in accordance with annual plans of surveillance (1), disposed by CE decisions (2002/649/CE; 2004/111/CE; 2005/464/CE; 2006/101/CE; 2007/268/CE; 2009/437/CE). The main objective of plans is the early characterization of outbreaks of influenza (1, 2). Already in november 2005 an early detection plan started on wild birds, developed by biologists, veterinarians and epidemiologists from IZS PLV with the coordination of Direzione Sanità Pubblica del Piemonte and Ce.R.M.A.S.. Moreover a well coordinated activity has been possible thanks to the collaboration of Comprensori Alpini, Ambiti territoriali di Caccia, Parchi and the Dipartimento Regionale di Caccia e Pesca. All the informations collected were used to set up a database arranged by CE and managed by National Reference Centre (IZS Venezie). The project is coordinated among the groups to maximize sampling of different species of birds in different locations avoiding duplication of effort. Subtypes of influenza A virus have been identified in the wild birds populations, predominantly ducks, geese and shorebirds, the natural reservoir for influenza A viruses. While the influenza viruses does not usually cause clinical signs of disease in wild aquatic birds, severe illness may occur when some of these viruses, of the subtypes H5 or H7, can mutate into highly pathogenic strains after being introduced into domestic poultry farms. For this reason, both active and passive surveillance are needed to better prepare the farm industry for any incursion of HPAI viruses.

Materials and Methods

We employ tests approved by current directives (1, 2, 3, 4) for virological diagnosis: virus isolation by inoculation of S.P.F. embryonated chicken eggs, RT-PCR and/or rRT-PCR (type A, H5, H7, H9: validated protocols distributed by OIE, FAO and National Reference Centre for Newcastle Disease and Avian Influenza IZS Venezie). For serological diagnosis: hemagglutination inhibition test for the detection of hemagglutinin subtype (H1-H16). After serological and virological tests, all positive and inconclusive samples are sent to OIE, FAO and National Reference Laboratory for Newcastle Disease and Avian Influenza -IZS Venezie for confirmation tests and subsequent potential typing, as established by national and international regulations (1, 2).

Results

A total of **1903 samples**, from the monitored taxonomic groups, were collected and tested in our Laboratory from 2006 to July 2009. Data reporting has been worked out on the basis of membership Order and monitored Species, relative number of samples collected and analysed matrix kind. Unlike 2006, in which there wasn't positive case for avian influenza, between July and September 2007 were isolated on eggs or revealed by RT-PCR or rRT-PCR **five influenza viruses** from wild duck; two of these (cloacal swabs) were characterized only on the basis of **type A,** in absence of eggs isolation; for the others, replication on eggs and relative isolation have been possible with subsequent subtyping (**H10N1** from one sample of organ pool, **H3N8** from one sample of organ pool and cloacal swab**, H5N2 LPAI** from one sample of cloacal swabs). In our Laboratory we have also highlighted occasional antibody positive cases anti-H7 and anti-H5 in wild duck serum. In August 2008 subtype **H4N6** from two cloacal swabs of two captured ringed wild ducks was isolated. In July 2009 we had one positive case for avian influenza from Mallard's cloacal swabs, belonging to **H7 LPAI** subtype. Moreover the use of embrionated eggs allowed the isolation of some strains of **paramyxovirus 2, 4 and 1** (Newcastle Disease Virus-NDV) (5).

Discussion and conclusions

During the four considered years, we observed an increase of active monitoring samples obtained from hunted species and from species captured for ringing (fact of great value, required by CE for epidemiological purposes in the event of recapture/retrieval), coordinated by National Institute for Wild Animals (INFS); therefore we also observed that the veterinary put higher attention in describing animal status, both on ringed/founded with ring subjects and on not ringed ones. The forwarding of organs, collected from dead founded animals decreased noticeably even if, according to recent studies, passive monitoring was found to be more efficient than to live bird surveillance when to detect HPAIV H5N1 in wild birds (6). Monitoring wild birds, particularly in Piedmont, appears equally distributed on the whole regional territory with numerically higher sampling than those foreseen by National Monitoring Plan (1); moreover a good representativeness has been seen either of species considered as reservoir by National Plan and of those defined sensitive to influenza virus, that may get in touch with domestic species or with wild species at high risk (1). During 2007, on the whole national territory **47 positive birds** for **17 different influenza viruses subtypes**, were detected in eight different bird species, seven of which belonging to Anseriformes Order (mute swan, black swan, wigeon, wild duck, garganey, common teal and Northern shoveler) and one belonging to the Charadriiformes Order (black-headed gull) (data OIE, FAO and National Reference Laboratory for Newcastle Disease and Avian Influenza -IZS Venezie).

Surveillance on wild birds may therefore perform its double aim: to verify circulation and prevalence of different avian flu viruses subtypes, including those not subjected to notification (LPAI), and to asses the presence/absence of notification subjected strains (HPNAI and LPNAI) in wild birds target populations.

Table 1- Positive isolates for orthomyxo and paramyxo :

Recognized subtypes	Species	Samples typologies	Month/Year from 2006 to 2009
NDV pathogen	Robin	Organ Pool	January 2006
Paramyxovirus 2	Grey Partridge	Cloacal swab	January 2006
Paramyxovirus 4	Wild duck	Cloacal swab	September 2006
NDV not pathogen	Pigeon	Organ Pool	October 2006
NDV not pathogen	Green-winged teal	Cloacal swab	November 2006
NDV pathogen	Pigeon	Organ Pool	December 2006
NDV pathogen	Pigeon	Organ Pool	February 2007
NDV pathogen	Pigeon	Organ Pool	April 2007
NDV not pathogen	Mallard	Cloacal swab	June 2007
Influenza type A	Wild duck	Cloacal swab	June 2007
Influenza type A	Wild duck	Cloacal swab	June 2007
H10N1	Mallard	Organ Pool	Augus 2007
H3N8	Mallard	Organ Pool	August 2007
H5N2(LPAI)	Mallard	Cloacal swab	September 2007
NDV pathogen	Pigeon	Organ Pool	January 2008
NDV pathogen	Pigeon	Organ Pool	May 2008
H4N6	Mallard	Cloacal swab	August 2008
NDV not pathogen	Wild-duck	Organ Pool	September 2008
Paramyxovirus 4	Mallard	Organ Pool	October 2008
NDV pathogen	Turtle-dove	Organ Pool	January 2009
NDV pathogen	Pigeon	Organ Pool	February 2009
H7 (LPAI)	Mallard	Cloacal swab	July 2009

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PCR SCREENING OF MAMMALIAN PREDATORS (CARNIVORA) FOR ADENO- AND HERPESVIRUSES

Eszter DANDÁR[1], Andor DOSZPOLY[1], Máté JÁNOSKA[1], Miklós HELTAI[2], László SZABÓ[2], Mária BENKŐ[1]

[1]*Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary;*
[2]*Institute for Wildlife Conservation, Szent István University, Gödöllő, Hungary*

Keywords: adenovirus, herpesvirus, nested PCR

Introduction and Objectives

Wide-spread occurrence of adeno- and herpesviruses in mammalian hosts has been well-documented by numerous serological surveys, the results of which are now being confirmed by consensus PCR screenings of different samples. Compared to the wealth of virus types recognized in the families of *Adeno-* and *Herpesviridae*, relatively few such viruses are known from mammalian predators i.e. carnivores.

Both viruses occur in a very wide palette of vertebrate hosts including fishes, amphibians, reptiles, birds and mammals. Moreover, herpes-like viruses have been isolated even from invertebrate hosts, albeit these viruses, as well as amphibian and piscine herpesviruses are now classified into novel families (*Malaco-* and *Alloherpesviridae*, respectively) contained in the new order *Herpesvirales* (1).

Although distinct cat and dog herpesviruses have been recognised, the merely two canine adenovirus types seem to have broader host range including foxes, bears, wolves, coyotes, racoons, etc. It is puzzling that, in spite of targeted attempts, no specific feline adenovirus could be identified to date. Thus the theory on host-virus co-speciation or co-evolution seems to be not, or only marginally, valid in case of carnivores.

The primary aim of this work was to screen samples from different species of mammalian predators for the presence of adenoviruses. Every sample was also tested for herpesviruses.

Materials and methods

Altogether 125 samples were collected from different sources without selection criteria other than carnivorous origin. Internal organs were taken from animals found dead in the wild without any history, or from individuals that had died in captivity with clinical signs of disease. Fresh excrements were also collected from apparently healthy zoo animals. An overwhelming part of the samples originated from Hungarian golden jackals (*Canis aureus*) that had been shot in hunts. One or more samples were examined from the following species: tiger (*Panthera tigris*), Eurasian lynx (*Lynx lynx*), domestic cat (*Felis catus*), serval (*Leptailurus serval*), red fox (*Vulpes vulpes*), grey wolf (*Canis lupus*), bush dog (*Speothos venaticus*), Indian dhole (*Cuon alpinus dukhunensis*), spotted hyena (*Crocuta crocuta*), brown bear (*Ursus arctos*), ring-tailed coati (*Nasua nasua*), polar bear (*Ursus maritimus)*, red panda (*Ailurus fulgens*), Eurasian badger (*Meles meles*), beech marten (*Martes foina*), and Oriental small-clawed otter *(Aonyx cinereus).*

Following DNA extraction, we used a nested PCR targeting the DNA polymerase gene. In case of both viruses, a very sensitive method, using highly degenerate consensus primers, have been elaborated (2, 3). In the herpesvirus genomes, the gene of glycoprotein B is adjacent to that of the DNA polymerase, and can also be detected by PCR. The nucleotide sequence of the products of positive reactions was determined, and used in phylogenetic calculations. Although the size of the DNA fragments amplified by PCR is relatively small (approximately 300 bp from the adeno-, and 200 bp from the herpesviruses), their sequence is generally suitable for a tentative genus classification of the detected virus.

Results

For the presence of adenovirus, only two samples from golden jackals were found to be positive. According to the sequence analysis of the PCR product, both samples contained a virus identical with canine adenovirus type 2 (CAdV-2).

Detection of herpesviruses was successful also in two samples only, which originated from a badger and from an Oriental small-clawed otter. The nucleotide sequence of the virus detected in the sample of the badger was almost identical with the corresponding sequence of the *Mustelid herpesvirus 1* (MuHV-1) belonging to genus *Percavirus* within the subfamily *Gammaherpesvirinae* (4). The virus we identified in the sample of the small-clawed otter seemed to be a novel herpesvirus genetically closely related to MuHV-1.

Discussion and conclusions

The very few positive samples, found in cases of both viruses were somewhat unexpected and disappointing. One explanation for this result could be the way the samples had been collected. There were many stool samples from healthy zoo animals, which obviously do not shed regularly either virus.

The two samples positive for adenovirus originated from golden jackals, a formerly extinct species in Hungary. Re-settlement of jackals started a couple of decades ago. Among the samples studied in this work, the specimens from golden jackals constituted the largest share of the samples. The presence of CAdV in different wild living carnivores including jackals has earlier been detected by serological surveys. However, this is the first report on the molecular detection of CAdV-2 in this host. Although the PCR method we used is rather sensitive, one probably needs to catch an individual in the condition of viraemia to have positive reaction. Our results further confirmed CAdVs being exceptional adenoviruses with a broad host spectrum (5).

Interestingly, also two samples were found to be positive for herpesviruses out of the 125 tested ones. However, one of the two positive samples was from the single badger we examined. Again, it is very likely that this animal had an active viraemic period, contributing perhaps to the road traffic accident which had killed the badger. Detection of sequences identical to those known from MuHV-1, strongly indicate that this is a genuine badger herpesvirus common not only in the territory of UK, but also in Central Europe (4). Detection of a closely related herpesvirus in the Oriental small-clawed otter supports the common taxonomical classification of gammaherpesviruses of carnivores. This particular animal died in the zoo, and had obviously a systematic herpesvirus infection. Normally, when in a latent phase, there is little chance to detect herpesviruses by PCR.

Further study, with PCR and sequencing, of the putative novel herpesvirus from the Oriental small-clawed otter is planned. Amplification of a part of the glycoprotein B gene was successful. Attempts to join the two partial sequences by PCR with specific primers are now in progress.

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FEASABILITY STUDY OF A CAPTURE AND TESTING SYSTEM FOR WILD BIRDS TO SET UP AN ''EARLY WARNING SYSTEM'' FOR WEST NILE VIRUS IN HORSE AND BIRD IN BELGIUM

Joëlle DE VRIESE[1], Olivier PONCIN[2], Bénédicte LAMBRECHT[1], Didier VANGELUWE[2], Thierry VAN DEN BERG[1], Carine LETELLIER[1]

[1]*Veterinary and Agrochemical Research Centre, Virology, Brussels, Belgium;*
[2]Royal *Belgian Institute of Natural Sciences (RBINS)*

Keywords: West Nile virus, wild bird monitoring

Introduction and objectives

West Nile virus (WNV) is a neurotropic flavivirus which was first isolated in the West Nile province of Uganda in 1937. Until the end of the 1990s, West Nile (WN) disease was considered as a minor risk for humans and horses because it only appeared sporadically. Since 1996, the year of the first large outbreak in Romania, and after the surprising detection of WNV in New York City in 1999, WN fever has become a major public health and veterinary concern in Europe and in the Mediterranean Basin, and in the USA. Aetiology of the disease indicated birds as the likely introductory and amplifying hosts with ornithophilous mosquitoes. Towards the evolution of the dispersal of the disease in Europe, we cannot exclude that the disease arises in our country, the populations of vector mosquitoes and the ecological conditions being present there. To monitor the presence of WNV in Belgium, an epidemiological surveillance program in wild birds and horses will be designed based on 2 levels: 1) passive surveillance, based on the investigation of case of abnormal mortality of birds and\or equines encephalitis and, 2) active surveillance based on the serological follow-up of poultry sentinels, wild birds and horses which will allow to detect a viral circulation in the absence of abnormal avian mortality. The objectif of this study was to set up capture and WNV detection systems for migratory passerines and corvids.

Materials and methods

Serological tools**:** The development of serological tests requires reference polysera. In order to obtain these, 5 week old SPF chickens were inoculated with 10^5 $TCID_{50}$/ml WN virus strain Is98. The chickens were bled at 2 and 3 weeks after a priming and at 6 weeks after 2 boosts with the same dose of inoculum.

The serological tests include seroneutralisation assay and ID-Vet ELISA kit (ID Screen® West Nile Competition). Using WNV strain IS98 seroneutralisation assay was performed following standard protocol (Niedrig et al., 2006).

Molecular tools**:** RNA was extracted from swabs and brain homogenate extracts with the Highpure kit (Roche). Two-step real-time PCR was performed by using published primers and probe targeting 3'UTR region (Lanciotti et al., 2000).

Active monitoring for wild birds**:** During April and to a lesser extend May and June 2008, 70 sylviidae belonging to 7 species were sampled (oral swabbing) in spring migration in Belgium.

Serological samples and oropharyngeal swabs were taken on Corvidae. Specific baited trapping cages (18 m^3) allowing to catch Corvidae were established in Brabant wallon and Liège provinces but also in the centre of Brussels on the IRSNB top. Additionally individual traps were experimented in two provinces from Belgium in order to capture breeding territorial pairs (fig 1).

Passive monitoring for wild birds**:** The target species were the Corvidae as they were considered as indicator of WNV circulation (Komar et al., 2003). Experimental system of wild bird carcasses collection was organized since February 2008 in collaboration with 2 non-profit organizations in two provinces running bird hospital for the establishment of a system of corpse's collection, labelling, storage and transmission.

Fig 1. Automatic trap for catching territorial crows (photo RBINS)

Results

The seroneutralisation assay was validated using at once equine sera from national laboratory reference (AFSSA - France) and reference chicken polyserum. This test allowed to highlight seroconversion in avian serum harvested 12 days after infection as well as to confirm high titers of neutralising antibodies in serum harvested 40 days after infection.

In order to validate the commercial ELISA kit, the same equine and chicken reference sera were used. Moreover, equine sera, routinely tested at VAR for other equine diseases in the context of AI centers or export were tested validating also this ELISA. Eighteen horses sera scored positive in ELISA and seventeen were confirmed as positive by seroneutralisation.

The repartition of the 59 Sylviidae swabs tested by RRT-PCR for the active monitoring is presented in table 1. None of the swabs was detected positive. The second objective was to studying the possible circulation of WNV in our regions. In this aim, samples sera from Corvidae, Carrion Crow (Corvus corone) and Jackdaw (Corvus monedula) were tested by the commercial competition ELISA. Two out of 86 sera gave doubtful result by commercial Elisa, however negative in the seroneutralisation test and the 84 other sera were ELISA negative.

For the passive monitoring, no suspect mortality event was recorded and the system of carcasses collection allowed us to analyzed virologically 27 brains of corvidae. The detection of WNV in brains by RRT-PCR did not show any positive result.

Table 1: Number of Sylvidae investigated for WNV infection

Species	*Number pos/total*
Sylvia atricapilla	*0/44*
Phylloscopus collybita	*0/1*
Sylvia borin	*0/1*
Sylvia curruca	*0/1*
Phylloscopus trochilus	*0/1*
Acrocephalus scirpaceus	*0/10*
Sylvia communis	*0/1*

Discussion and conclusions

The active monitoring aims to study the possible introduction of the WNV in Belgium. So the monitoring consists first in studying the feasibility to take oropharyngeal swabs on passerines soon after they return from African wintering area where WNV is endemic. A few weeks before trapping and sampling the birds concerned were in the Mediterranean basin or sub-Saharan Africa (west or east). After adapting swabs type and swabbing technique, no problems were encountered for oropharyngeal liquid sampling on small passerines. Corvids were easily trapped and dead birds were collected. To date, no evidence of WN infection has been detected. The samlpling of wild bird will now be extended to set up a surveillance system.

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REOVIRUS RELATED PATHOLOGICAL LESIONS AND CONSEQUENTIAL DEATH IN ROUGH GREEN SNAKE (*OPHEODRIS AESTIVUS*)

János GÁL[1], Míra MÁNDOKI[1], Miklós RUSVAI[1], Judit TAVASZI[2], Szilvia FARKAS[3]

[1]*SzIU-Faculty of Veterinary Science, Department of Pathology and Forensic Veterinary Medicine, Budapest;*
[2]*Lab-Research Ltd., Veszprém;*
[3]*Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest*

Keywords: cytophatic changes, nested PCR, reovirus infection, snake

Introduction and objectives

Reoviruses have a non-enveloped, icosahedral capsid about 70 nanometres across containing a core of segmented, double-stranded RNA. The *Reoviridae* family is divided into several genera, one of them is the *Orthoreovirus* genus. The members of the Orthoreovirus genus were isolated from several different mammalian, avian and reptile species such as snakes (1, 2, 4, 7, 8), lizards (5) and turtles (6).

Reptilian reoviruses induce cytopathic effect with giant cell formation (3) similarly to bird reoviruses, the Nelson Bay bat reoviruses and the baboon reovirus causing encephalitis. All reoviruses identified up to now belong to the orthoreovirus genus (3, 8).

Reoviruses most often cause respiratory signs (labored breathing with breathing sound) and central nervous symptoms (involuntary movement, lack of coordination), but in some cases the infected reptiles die without any previous signs (5, 6). Pathological lesions developed in the liver as result of the viral infection are vacuolar degeneration of the hepatocytes, appearance of necrotic foci (4, 6), but sometimes enteritis is also observed (6). The respiratory symptoms can be explained by interstitial pneumonia and tracheitis. In turtles (*Testudo graeca*) lingual ulceration occurred (6).

Clinical signs and pathological lesions are not pathognomonic for reoviruses, as paramyxovirus infection of snakes results in appearance of similar lesions. Reo- and paramyxo viruses are also alike on tissue cultures because both of them are causing cell fusion and syncytium formation (1, 2, 7, 8). Supplementary investigations (e.g. PCR, virus isolation) are needed to identify the pathogen.

Materials and methods

During the autumn of 2007 several Rough Green Snake carcasses imported from the United States were brought to the SzIU Faculty of Veterinary Science for pathological investigation.

The mortality occurred during the quarantine period after the arrival of the snakes. The animals rejected the food (cricket, wax worm, grub), hours before death showed signs of incoordination and took breath with opened mouth.

The carcasses were dissected and samples were collected from the parenchymal organs showing pathological lesions and were stored in 4% neutral buffered formaldehyde solution or at -80˚C for histological and PCR examination, respectively. Liver, intestine and lung samples were taken for virological examination. The tissue pieces were homogenized and the RNA was extracted with Trizol. First reverse transcription was performed to get cDNA, than the desired gene region was amplified in a nested PCR reaction. The amplified products were visualized, cut from the gel, extracted and sequenced. The homogenized tissue solutions were cultured on VH-2 cell line for virus isolation.

Results

External examination revealed sunken eyes due to exsiccosis. During dissection the fat depots were shrunken because of emptying. The liver appeared bright yellowish, the stomach and the intestines contained small amount of mucoid content. Mild hyperemia occurred in the lungs. Other organs did not show any macroscopic lesions.

Histopathological examination of the organs showed pathological simple fatty infiltration in the liver, hyperemia, edema, and moderate inflammatory cellular infiltration in the lung. Unobtrusive amount of uric acid was deposited in the lumen of the renal tubules.

Nucleotide sequences were identified, aligned to each other, and to the reference strain and the amplicons showed the highest identity with the nucleotide sequence of the reoviral genome. The phylogenetic analysis of the partial sequences proved that the viral genomes belong to the Orthoreovirus genus.

In the cell cultures cytopathic changes were observed which are typical for the orthoreo genus such as syncytium formation, detachment of cells, cell degeneration and necrosis (Figure 1.).

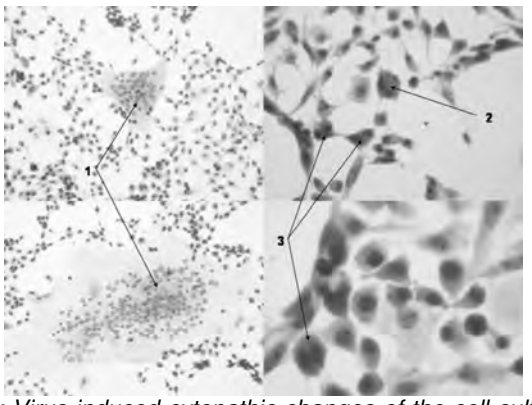

Figure 1: Virus induced cytopathic changes of the cell culture on VH-2 cell line; 1. syncytium formation, 2. karyorrhexis, 3. mitosis

Discussion and conclusions

In conclusion we state that the pathological and histological lesions found in insectivorous Rough Green Snake developed due to orthoreoviral infection demonstrated by PCR and isolation on VH-2 cell line.

According to our knowledge diseases of Rough Green Snakes related to orthoreoviral infection were not described before, so that is the first documentation of the appearance of orthoreovirus in this species.

Presenting this case we would like to draw attention to the risks of spreading infectious diseases with the international trade and import of captured reptile species.

Acknowledgements

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NOVEL SIMIAN ADENOVIRUSES – COMPARISON WITH FORMERLY ISOLATED PRIMATE ADENOVIRUSES

Máté JÁNOSKA, Andor DOSZPOLY, Győző L. KAJÁN, Laura PANTÓ, Balázs HARRACH

Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary

Keywords: nested PCR, simian adenovirus, new world monkey, phylogeny

Introduction and Objectives

Adenoviruses are double stranded DNA viruses, infecting a wide range of vertebrate hosts, from fish to mammals. Twenty-five simian adenovirus (SAdV) serotypes have been identified (SAdV-1 to 25). The first 20 of these originate from Old World monkeys (*Cercopithecidae*), while SAdV-21 to 25 were isolated from chimpanzee (*Pan troglodytes*). A further chimpanzee strain called C2 was described as a probable new type. Four groups were formed from the first 16 SAdV types based on the hemagglutination properties (Rapoza, 1967). Human adenoviruses (HAdV) are classified into 6 species (*Human adenovirus A* to F). The seventh proposed species, named Human adenovirus G is awaiting final ratification. Into some HAdV species, monkey or chimpanzee AdVs are also classified (Benkő et al., 2005). A further officially accepted species (*Simian adenovirus A*) contains only monkey AdVs. Various molecular data are available of these viruses. Ten of the 25 serotypes have been fully sequenced (SAdV-1, -3, -6, -7, -20 to 25) while the available molecular data are limited of the remaining serotypes (mostly just the VA-RNA gene is sequenced, Kidd et al., 1995). In this study we planned to gain partial sequences from the prototype strains and performed a general screening for adenoviruses in simian samples available from Hungarian zoos. Furthermore, we tried to determine the phylogenetic relations between these viruses, using the resulted partial gene sequences.

Materials and methods

The simian samples were from Hungarian zoos, and we tested both stool (24) and liver (22) samples during the general screening. This was performed using a nested PCR method targeting the DNA polymerase gene of the adenoviruses (Wellehan et al., 2004). We tested this method on the SAdV isolates originating from the ATCC (American Type Culture Collection), too. Further primers were designed based on the primate adenovirus IVa2 and penton base gene regions. These methods were tested on the prototype strains and on the positive simian samples. The nested approach was important because the low viral DNA concentration in the non-isolated samples. The positive samples were sequenced using the inner primers of the reactions. The gained sequences were aligned using MUSCLE. The phylogenetic calculations were performed online using the PHILYP package (PROTDIST, categories model; FITCH, global rearrangements) available on the MOBYLE webserver (http://mobyle.pasteur.fr).

Results

At least two partial genes were obtained from the prototype strain collection of the Old World monkey adenoviruses (SAdV-1 to 19). The chimpanzee C2 strain is related to HAdV-B. Out of the 46 zoo samples, four were found positive. A novel virus was detected in the sample of two New World monkeys: red-handed tamarin (*Saguinus midas*) and golden-headed lion tamarin (*Leontopithecus chrysomelas*). The additional positive samples were stools from gorilla (*Gorilla gorilla*) and orangutan (*Pongo pygmaeus*). These contained novel AdVs clustering close to HAdV-B and C types, respectively.

Discussion and conclusions

The virus originating from the New World monkeys represents the oldest lineage of the simian adenoviruses. This result supports the co-evolution theory on the adenoviruses (Benkő and Harrach, 2003). This is the first adenovirus detected in New World monkeys, so we plan to collect as much molecular information about this virus as possible. However, these partial New World monkey AdV sequences propose a need for the establishment of a new simian adenovirus species. By applying the gained partial sequences from the other SAdVs, it seems to be possible to make a preliminary classification for them. Most of them seem to belong to earlier established AdV species while some may be members of possible new species. (Fig. 1). Our data support quite well also the old classification based on the haemagglutination data and VA RNA gene(s).

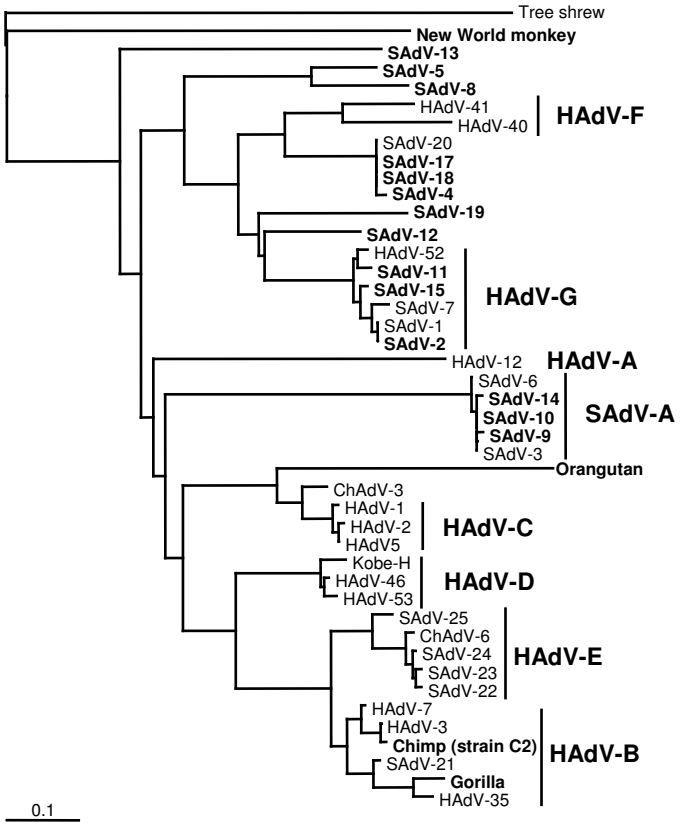

Figure 1: Phylogenetic tree of the partial adenoviral DNA polymerase gene. The results of this work are indicated as bold. The species names are written as bold and using bigger fonts.

Acknowledgements

This work was supported by NKTH-OTKA K67781 and OTKA K72484 grants.

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COMPLETE SEQUENCE AND GENETIC FEATURES OF RAPTOR ADENOVIRUS 1: A NOVEL, NON-ISOLATED SPECIES IN THE GENUS *SIADENOVIRUS*

Endre R. KOVÁCS, Balázs HARRACH, Mária BENKŐ

Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary

Keywords: siadenovirus, raptor adenovirus 1, genome sequencing, PCR, phylogeny

Introduction and Objectives

Adenoviruses constitute a family of double-stranded DNA viruses of medium size, diverse on the genomic level. The genus *Siadenovirus* contains species with the shortest genomes (~26 kb) within the family, such as those of the turkey hemorrhagic enteritis virus (THEV) and the frog adenovirus 1 (FrAdV-1) (1). These two species were the only completely sequenced viruses in the genus for a decade. Organ samples of three different birds of prey were suspected to be infected with adenovirus and subjected to PCR testing (2), which yielded a novel type of siadenovirus (3). Repeated attempts at isolation were made without success. As the new type represented a new species in a rare genus, the main objective of the study was to acquire the complete genomic sequence by way of performing PCR on the extracted total DNA of the organ samples.

Materials and methods

DNA was extracted from liver scrapings of three individual raptorial birds of three species (Bengal and Verreaux's eagle owl and Harris hawk). PCR was performed on the pooled sample using three different types of primers: 1. genus-specific degenerate consensus 2. genus-specific non-degenerate 3. raptor adenovirus specific (4). Primers were paired in all possible combinations and all reactions were optimized for each primer pair and DNA polymerase in order to amplify fragments of the viral genome. At least three types of high-fidelity thermostable DNA polymerases were used in the reactions (REDTaq®, Phusion™, FailSafe™). Sequencing was performed with the BigDye® Terminator v3.1 Cycle Sequencing Kit and ABI 373A automated DNA sequencers (APPLIED BIOSYSTEMS). Longer amplicons (>0.8 kb) were subjected to primer walking. Fragments were assembled into a single continuous sequence by means of the Staden sequence analysis package. Phylogenetic reconstruction was done by way of distance matrix analysis using tools in the PHYLIP package (5).

Results

The complete sequence of the raptor adenovirus 1 (RAdV-1) genome was derived and found to be 26 282 bp in length. Base composition proved to be fairly biased in favour of the A+T dinucleotide (61.5%), however, A+T content of the gene pVII is balanced (51.6%) relative to that of all other genes in the genome. Inverted terminal repeats were found to be the shortest (30 bp) within the adenovirus family. Genome organisation is consistent with that of formerly sequenced siadenoviruses: 17+1 conserved genes arranged centrally in the linear genome, and 5 genus-specific ORFs located at both ends, namely sialidase, ORFs 4, 7, 8 and E3. No function has been attributed to any of these ORFs within the genus. Sialidase bears relation to bacterial neuraminidases. ORF4 is present in THEV and RAdV-1, but is not conserved in FrAdV-1. About 30 codons downstream is an ORF that, if functional, codes for a hydrophobic protein and is conserved in all three species. The single ORF in the 'E3' region is unrelated to mastadenovirus E3 proteins. ORFs 7 and 8 are located in the right end. Splice sites were found in the genes pTP, DBP and 33K. U exon, which is only conserved within the genera but not throughout the family, is present, whose 3' exon could not be located. No RGD motif could be found in penton base. This motif, besides the essential fiber–CAR interaction, is one of the prerequisites of entry via binding to cellular α-integrins, but not in enteric adenoviruses such as raptor adenovirus 1.

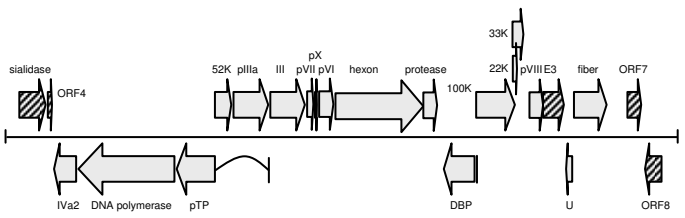

Figure 1. Genome layout of raptor adenovirus 1. Dashed arrows indicate the five genus-specific genes.

Discussion and conclusions

We report the complete sequence, phylogenetic and taxonomic place of RAdV-1 within the genus *Siadenovirus*, formerly represented by only two species. The adequate phylogenetic distance of RAdV-1 from TAdV-3 and FrAdV-1, and the different host range (hawks and owls contra frogs and galliforms) confirm the proposal of establishing the new species Raptor adenovirus A. The discovery and complete molecular analysis of RAdV-1 is important in order that we gain a better understanding of the minimal adenoviral genome.

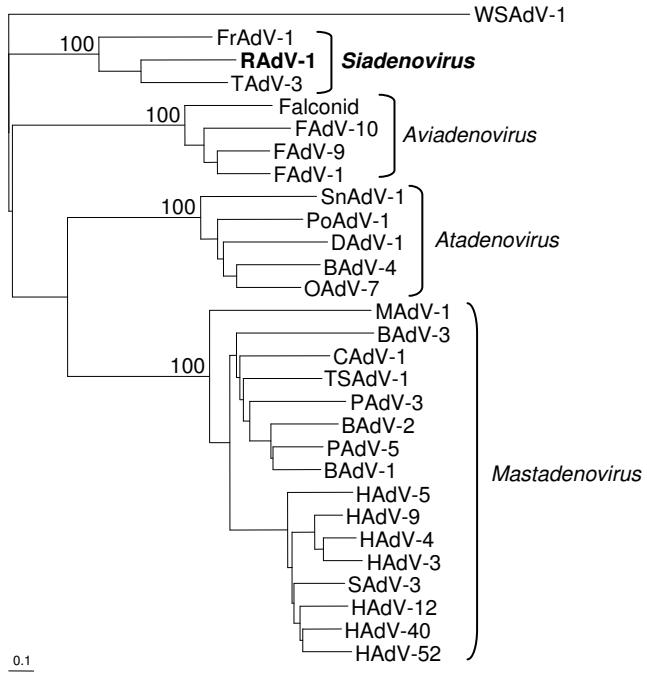

Figure 2. Phylogenetic tree constructed by distance matrix analysis from aa sequence alignments of the main capsid protein hexon. B, bovine; C, canine; D, duck; F, fowl; Fr, frog; H, human; M, murine; O, ovine; P, porcine; Po, possum; R, raptor; S, simian; Sn, snake; T, turkey; TS, tree shrew; WS, white sturgeon.

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PHYLOGENETIC CHARACTERISATION OF CANINE DISTEMPER VIRUSES DETECTED IN NATURALLY INFECTED WILD AND DOMESTIC CARNIVORES IN NORTH EAST OF ITALY

Isabella MONNE, Annalisa SALVIATO, Carlo CITTERIO, Mariapia COVA, Marco BREGOLI, Paola DE BENEDICTIS, Claudio PASOLLI, Elena MAZZOLINI, Viviana VALASTRO, Giovanni FARINA, Karin TREVISIOL, Giovanni CATTOLI

Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padova, Italy;
Keywords: Canine distemper virus, Carnivores, Italy

Introduction and Objectives
Canine Distemper virus (CDV) belongs to genus *Morbillivirus* and it causes a severe viral disease affecting a broad range of wild and domestic carnivores. The disease is mainly characterized by nervous, respiratory and gastrointestinal signs. Since 2006, a CDV epizootic has involved wild carnivores in the North East (NE) of Italy. In addition, several CDV cases have been reported in domestic dogs from the same geographic area between 2000 and 2007. The genetic variation of the gene encoding for the hemagglutinin protein (H gene) allows distinction of various CDV lineages according to geographical origin and animal host (3). There are no recent reports describing the phylogenetic characteristics of the CDV viruses causing the severe distemper cases in wild carnivores in Italy and it is unclear whether the CDV detected in the domestic dogs are genetically related to the distemper viruses circulating in the wild life species. The objective of our study was to analyse phylogenetically the H gene sequences obtained from 29 CDV positive samples detected in wild and domestic carnivore population from NE of Italy.

Materials and methods
The H gene of 29 canine distemper viruses representative for geographical area of origin and date of collection were sequenced in this study. 19 out of 29 strains were identified in samples obtained from wildlife carnivores shot or found dead in the NE area of Italy. In particular distemper viruses identified from 11 foxes (Vulpes vulpes) and 8 badgers (Meles meles) were included in this analysis. The remaining 10 samples were collected from domestic dogs from rural and urban areas of the NE regions. RNA was isolated from organs (lungs and brains) using the Nucleospin RNA II kit (Macherey Nagel) according to manufacturer's instructions.
The amplification of the H gene was obtained by RT-PCR using gene-specific primers (available upon request). PCR products were purified (ExoSAP-IT, USB, Cleveland, Ohio, USA) and sequenced in a 3130xl Genetic Analyser (Applied Biosystems). Phylogenetic analysis was performed using the neighbour-joining method in the MEGA 3 programme (2). Nucleic acid sequences of the H gene obtained in the present study were compared with the respective gene sequences of CDV strains available in the public database of NCBI.

Results
Phylogenetic analysis of the sequences obtained for the present study revealed that the CDV collected in the North East of Italy cluster in 2 distinct lineages. All the distemper viruses detected from wildlife species belong to the European lineage (3). This lineage was already identified in Italy from domestic dogs and is mainly composed by CDV circulating in Europe. The sequences of the CDV collected from wild carnivores in North Italy were closely related to each other (similarity ranged between 99,4% and 100%) and the higher similarity (>99%) was found with the sequence of a CDV collected in Hungary from a domestic dog. Two distemper viruses, identified respectively in 2000 and 2007 from domestic dogs living in the NE Italy, grouped within this lineage but clustered in two separate branches of the phylogenetic tree. The remaining 8 strains identified from the domestic dog population of this area were located on a separate ramification of the phylogenetic tree. In particular these sequences fall in the Arctic lineage (3) previously reported in 2 distemper episodes in Southern Italy. This lineage was originally detected in the susceptible population of the Arctic ecosystem and it was recently recognised in Eastern Europe (1).

Discussion and conclusions
CDV strains detected from wildlife species of NE Italian origin between 2007 and 2009 displayed a high genetic homogeneity within the European lineage. This lineage was identified also in dogs sampled in the NE of Italy suggesting that the CDV strains detected in wild and domestic carnivores of this area shared a common ancestor. However, the high similarity found between the wildlife distemper viruses collected in a relatively long period (from 2007 to 2009) underlines that no further viruses were introduced in the wild carnivores population and that an established genetic cluster of the virus is circulating between foxes and badgers in the NE of Italy.
The repeated identification of the Arctic lineage in Northern Italian dog population underlines that the previous description of this lineage in South of Italy was not an occasional finding. The Arctic lineage is composed by distemper viruses originally detected in Greenland, North America, China and Siberia. The means by which this lineage was introduced in Italy remains unclear but it can be suggested that intense trade of dogs involving several European and non-European countries may be considered an explanation. Based on the results of this study it appears that the Arctic like distemper virus has become an established CDV lineage of the Italian dog population.

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INVERTEBRATE IRIDOVIRUS INFECTIONS IN INSECTIVOROUS PETS? QUEST FOR A BETTER DIAGNOSTIC METHOD

Tibor PAPP, Dirk SPANN, Rachel E. MARSCHANG

Institut für Umwelt- und Tierhygiene, Hohenheim University, Garbenstr. 30, 70599 Stuttgart, Germany

Keywords: diagnostics, host-switch, iridovirus, pet, reptile

Introduction and objectives

The family *Iridoviridae* consists of five accepted genera: two of these (*Lymphocystivirus, Megalocytivirus*) contain viruses of fish, one (*Ranavirus*) has members from all cold-blooded vertebrates, including reptiles and two (*Iridovirus, Chloriridovirus*) were known to be arthropod viruses. However, the invertebrate iridoviruses (IIVs, genus *Iridovirus*) have recently also been described from lizards in Germany by independent research groups (2,4). Since then we have detected invertebrate iridoviruses (IIV) from six different lizard species, representing 4 different families. We have also detected IIV in scorpions that died suddenly at three different periods of time over the course of a year, all belonging to the same owner. Where available, the prey insects were analysed too. Diseased lizards have shown mainly skin problems with hyperkeratosis, however, IIVs were also detected from a tumor in the region of the femoral-pore of an agamid lizard. Scorpions have been reported to loose their UV-coloration before death. Crickets with high virus loads had bluish iridescence in their fat-bodies.

Preliminary characterization of IIV from these hosts by sequencing a portion of the highly conserved major capsid protein (MCP) gene has shown that these isolates are highly like (or identical) to one another other and to the recently described cricket iridovirus (GbIV) (3), a presumed variant of Chilo iridescent virus, the type species of the genus. These viruses propagate well on reptilian cell lines, as well as on insect ones.

Animal infection trials have been conducted with crickets, scorpions and lizards to discover the pathogenic potential of IIVs. Small portions of their large dsDNA genomes are also being sequenced to compare different isolates. Conventional PCRs, real-time PCR, and in-situ hybridisation (ISH) methods have been tested and developed for improved detection of IIV infection. Here, preliminary results will be presented from the ongoing research on the molecular characterisation of the different isolates and the comparison of different diagnostic methods.

Materials and methods

IIV isolates from agamid (*Pogona vitticeps*, *Uromastyx sp.*), chameleonid (*Chameleo quadricornis, Ch. hoehnelii*) and iguanid (*I. iguana*) lizards, from an emperor scorpion (*Pandinus imperator*) and from crickets (*Acheta domesticus*, *Gryllus spp.*) were propagated on reptilian cell lines (TH-1, VH-2, IgH-2) and concentrated by ultracentrifugation. 4 different genes (coding the MCP, DNA polymerase, exonuclease, thymidilate synthase, ATPase and intermediate early /IE/ proteins) were PCR amplified and sequenced with earlier described primers (1) and newly designed ones. Real-time PCR primers and probes were designed based on the obtained sequences.

Virus isolation, conventional PCRs and real-time PCR methods were compared in their sensitivities using 10 fold virus dilution rows. Plasmid cloned PCR products were used to determine the cutoff values of the different PCR methods.

Results

The divergence between the different IIV isolates was very low (under 1 %) for all partial gene sequences analyzed. Most of the isolates were absolutely identical with one another and with the with the GbIV partial MCP gene sequence, the few differing ones had only 1-2 silent mutations. The ATPase, thymidilate synthase and IE protein coding gene portions were all identical between the different isolates, and 95.7-98% or 92.4-100% similar with the homologous CIV genes (no GbIV data are available) for the nucleotide (nt) or amino acid (aa) sequences respectively (see Table 1).

The exonuclease and polymerase gene sequences showed the largest divergence compared to those of CIV, and insertions/deletions (6-20 bp) occurred in both genes. No variability was detected between isolates for the exonuclease gene, although there were up to 3 (2 non-silent) point mutation differences between the polymerase sequences from the various isolates.

	Exonuc-lease (012L) *	***Polyme-rase (037L)****	***ATPase (075L)***	***Thymidi-late synthase (225R)***	***MCP (274L)***	***Int. early proteine (393L)***
	1689 from 1878 nt	*700 from 3822 nt*	*630 from 777 nt*	*510 from 888 nt*	*All of 1404 nt*	*664 from 1365 nt*
DNA	*90.0%**	*93.6%**	*97.9 %*	*95.7%*	*97.8%*	*98.0%*
PROT.	*90.6%**	*92.5%**	*100 %*	*92.4%*	*98.5%*	*96.3%*

Table 1. Sequence identity values between the different genomic regions of new IIV isolates from various hosts (consensus sequence) compared to CIV.
** = insertions/deletions are present*

A comparison of the different diagnoistic methods has shown that a consensus nested PCR detecting the MCP gene is ca. 100 times more sensitive than any other PCRs or the virus isolation method. A real-time PCR targeting the same gene has been developed, which has a similar sesitivity.

Discussion and conclusions

The low variation detected between the various isolates indicates that there is a single IIV species widely prevalent within the Eupean pet trade. Similar viruses have also been found in other labs and in other countries (e.g. Hungary, Farkas Sz, personal communication). The exonuclease and the polymerase genes are being further analysed and compared.

The long inapparent infection of prey insects makes a timely laboratory diagnosis of IIV infections crucial. The highly sensitive nested MCP PCR is eligible for this purpose. It is, however, very prone to contamination and often gives a multiple band pattern. The new real time PCR can hopefully sort out these problems. All samples obtained from our previous and from our ongoing animal infection studies are being tested by the real-time PCR as well.

The pathogenic potential of IIVs in lizards (and in scorpions) remains unclear. Hovewer, their continous occurence in various tissues, particularly in lizards with proliferating skin lesions makes the question interesting and further efforts are being made to clear this up.

Acknowledgements

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EXAMINATIONS AIMING AT THE VERIFICATION OF THE REPTILIAN ORIGIN OF ATADENOVIRUSES

Judit PÉNZES, Andor DOSZPOLY, Balázs HARRACH, Mária BENKŐ

Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary;

Keywords: adenovirus, co-evolution, nested PCR, reptile

Introduction and Objectives

Adenoviruses (members of the family *Adenoviridae*) are widely distributed among vertebrates. By the development of modern diagnostic methods, it has become possible to detect viral presence directly from the samples of the animals. By the extension of the research field, it turned out that in fact every major vertebrate class, from fish to mammals, may harbour adenoviruses (Benkő *et al*., 2005). The individual adenovirus types generally possess quite narrow host spectrum. Thus each animal species may likely have its own adenovirus. This makes adenoviruses very good subjects for investigating the co-evolution of viruses and hosts (Benkő and Harrach, 2003). Currently the family *Adenoviridae* is divided into four accepted and one proposed genera. The genus *Atadenovirus* is hypothesized to represent the lineage which supposedly co-evolved with reptiles. Interestingly, the firstly recognized members of this genus had been isolated from birds and ruminants. On the initial phylogenetic tree reconstructions, these adenoviruses occupied a distant, well separated branch, implying an ancient host origin. The first detailed genome analysis of an adenovirus isolate originating from snake fully supported the theory on the reptilian origin of atadenoviruses (Farkas *et al*., 2008). Detection of additional novel atadenovirus types in different lizard species confirmed the theory (Wellehan *et al*., 2004). The aim of this study was to uncover further evidences concerning the reptilian origin of atadenoviruses by screening different reptile and amphibian species for the presence of adenovirus.

Materials and methods

The samples for analysis were from animals that had died in local pet stores. Altogether 70 samples, including 37 from reptiles, and 33 from amphibians were examined. All the amphibian samples originated from anurans, while the reptile samples were from turtles, tortoises, lizards, and snakes. The necropsy findings were not relevant. Many animals were young and tiny in size. Moreover, the carcasses were often emaciated or dry. Samples were taken from the intestines, liver and spleen. The DNA was extracted by the use of Qiagene kit, and screened by a nested PCR method, which amplifies an approximately 300 bp long fragment from the adenoviral DNA polymerase gene (Wellehan *et al*., 2004). The nucleotide sequence of PCR products from the positive samples was determined and subjected to phylogenetic analysis. Sequences from samples of the same host species were compared in order to examine the variability of the virus.

Results

Out of the 70 samples, 12 were found positive. These included the samples of both bearded dragons (*Pogona vitticeps*) examined, as well as the sample of a corn snake (*Elaphe guttata*), a rat snake (*E. obsoleta*), a white-throated monitor (*Varanus albigularis*), five stumped-tailed pygmy chameleons (*Rampholeon brevicaudatus*), and two species of poison-dart frogs (*Dendrobates auratus, Phyllobates vittatus*). Five adenovirus types could be differentiated. The viruses found in the bearded dragon samples seemed to be identical at the amino acid level with the bearded dragon adenoviruses previously found in the United States (Wellehan *et al*., 2004), Germany (Papp *et al*., 2008), and Austria (Kuebber-Heiss *et al*., 2005). On the nucleotide sequence level, however, they were only identical with the virus detected in the United States in 2004. The sequences obtained from the two positive snake samples were identical with each other, as well as with the corresponding region of the DNA-polymerase gene of the fully sequenced snake adenovirus type 1, originating also from corn snake (Farkas et al., 2008). Identical adenovirus had previously been detected in the sample of a red-tailed boa (*Boa constrictor*) originating from Germany (Marschang *et al*., unpublished). Two novel sequences, indicating the presence of yet not described viruses, were determined from the sample of a white-throated monitor and of five pygmy chameleons. According to the phylogenetic tree reconstruction, all the adenoviruses, detected by us in reptilian samples, could be assigned to the genus *Atadenovirus.*

A fifth type of adenovirus was demonstrated in both of the positive samples of poison-dart frogs. Surprisingly, the putative new virus also clustered in the genus *Atadenovirus*, but in a common branch with the ruminant isolates, and not with the reptilian ones.

Atadenoviruses have originally been named after the biased (A+T rich) base composition, discovered in the genome of their first characterized representatives found in ruminant and avian hosts (Benkő and Harrach, 2003). Interestingly however, the G+C content of the DNA of snake adenovirus 1 (Farkas *et al*., 2008), as well as the short DNA-polymerase gene sequences of the putative lizard adenoviruses had balanced G+C content close to 50% (Wellehan *et al*., 2004). On the other hand the adenovirus demonstrated in the poison-dart frog samples had low G+C (38.4%) similar to that of the bovine, ovine and duck atadenoviruses.

Discussion and conclusions

Besides increasing the number and diversity of atadenoviruses detected in reptiles, this study revealed first time the presence of such viruses in amphibians hosts. The infection rate of the examined reptiles (27%) was much higher than experienced in other vertebrate hosts; however this can be partially due to the fact that naturally deceased individuals were tested exclusively. The infection of both bearded dragons examined might indicate that the European breeder colonies are all infected. The clinical sings caused by the virus infection are not very characteristic, but can result in serious losses. Attempts to establish adenovirus-free breeds are now being made in the United States.

The infection rate found in amphibians was significantly lower (6%). Yet, we consider it very important because to date, only one single adenovirus has been demonstrated from any amphibian hosts. The adenovirus isolated from Northern leopard frog (*Rana pipiens*) in 1973, has later been proven to be a siadenovirus (Benkő *et al*., 2005). Our finding is the very first detection of adenovirus infection in poison-dart frogs. The apparent clustering of this virus to the genus *Atadenovirus* is somewhat surprising, and urges the reconsideration of the co-evolution theory. However, the low G+C content, which is hypothesized to mirror inter-class host switches, imply that these frogs might not be considered as natural hosts.

Further examination of the three putative novel atadenoviruses (detected in the white-throated monitor, the pygmy chameleons, and the poison-dart frogs is of great interest.

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REAL TIME RT-PCR ASSAYS FOR MONITORING AVIAN INFLUENZA A H5/H7 VIRUSES IN WILD AND DOMESTIC BIRDS

Francesca RIZZO[1], Francesca SIDOTI[2], Cristina COSTA[2], Massimiliano BERGALLO[2], Rossana CAVALLO[2], Maria LUCIA MANDOLA[1]

[1]Veterinary Medical Research Institute for Piemonte, Liguria and Valle d'Aosta, S.C. Virology, Turin, Italy;
[2]Department of Public Health and Microbiology, Virology Unit, Turin, Italy

Keywords: avian, H5/H7, influenza A, Real time RT-PCR

Introduction and objectives

Type A influenza viruses are further divided into subtypes based on the antigenic relationships of the surface glycoprotein hemagglutinin (H) and neuraminidase (N): at present 16 H subtypes (H1-H16) and 9 N subtypes (N1-N9) have been recognised [1]. Influenza A viruses infecting poultry cause avian influenza (AI) and can be divided into two distinct groups: highly pathogenicity AI (HPAI) and low pathogenicity AI (LPAI). To date the virulent viruses are restricted to subtypes H5 and H7, subject to notification under the law. Currently, virus isolation (VI) in embryonating chicken eggs and subsequent H and N subtyping by serological methods constitute the standard for avian influenza viruses detection and subtype identification. These methods are highly accurate and sensitive, but also laborious and time-consuming [2]. Therefore, in addition to containment procedures, rapid and sensitive subtyping assays for early H5 and H7 diagnosis became absolutely necessary to reduce their spread and the risk of an epidemic with potential transmissions to humans [3].

Molecular assays are not only more rapid but also more sensitive and specific than conventional procedures. RT-PCR and real-time RT-PCR have previously been used to detect influenza A virus and for the specific diagnosis of H5, H7 and H9 viruses [4]. No primer and probe sets are currently available for the identification of all H5 and H7 isolates. In this study, we have developed specific and sensitive rRT-PCR assays able to detect all H5 and H7 isolates that have been isolated since 2000 worldwide. Moreover, we have also compared these molecular assays with the conventional VI in terms of sensitivity.

Materials and methods

Selected tritrated H5N2 and H7N3 subtypes of avian influenza viruses, were used for the standardization of the Real time RT-PCR assays and to test the sensitivity of these molecular assays compared with virus isolation.

Using 134 and 205 HA nucleotide sequences for subtypes H5 and H7 respectively, isolated worldwide since 2000 and available in the GenBank database, multiple sequence alignments genes were performed and both primers and probes were chosen using Primer Express Software Version 3.0. Because of the significant sequences variation of H5 gene, we branched viral subtype H5 into two clusters with specific different degenerate primers and with a common degenerate probe: H5N1 cluster, specific for H5N1 isolates and H5NX cluster, specific for H5N2, H5N3, H5N5, H5N6, H5N7, H5N8 and H5N9 isolates. To determine sensitivity of the real time RT-PCR assays, a clone containing the complete sequence for each target was used. The amplification products were cloned into the TOPO TA cloning vector and plasmids with the HA insert were propagated in competent Escherichia coli TOP10 cells. Viral plasmid dilutions (from 5×10^9 copies/µl to 5×10^{-1} copies/µl) were used to estimate the dynamic range of the plasmid. Using 10-fold serial dilutions of titrated AI viruses (from 10^2 to 10^{-4} EID_{50}/100 µl) we compared the molecular assays and gold standard VI in term of sensitivity.

To evaluate the diagnostic performance characteristics of the real time RT-PCR assays, we analyzed 114 samples from wild and domestic birds. The specimens consisted of 70 cloacal swabs from wild birds, 14 tracheal swabs from domestic birds, and 30 organ pools (18 from wild birds and 12 from domestic birds).

Results

To examine the dynamic range (linearity measurement that indicates reliability of real time RT-PCR assay quantification) of H5N1, H5NX and H7 genes quantification by real time RT-PCR, serial dilutions of the plasmid standard ranging from 10^{10} to 1 copies/reaction were carried out and real time RT-PCR assay was able to quantify from 10^{10} to 10 copies/reaction for all the H5N1, H5NX and H7 genes. The R^2 index for H5N1, H5NX and H7 genes was 0.991, 0.992 and 0.995, respectively and the standard curve slope was 3.033, 3.006 and 3.3, respectively. Sensitivity of real time RT-PCR assays is defined by the lowest concentration of target quantified at a frequency of 100%. H5N1, H5NX and H7 sensitivity was 1 copy/reaction, 100 copies/reaction and 10 copies/reaction, respectively. Limit of detection of real time RT-PCR methods, defined by the lowest concentration of target quantified, was 10^{-1} copies/reaction, 10 copies/reaction and 10 copies/reaction for H5N1, H5NX and H7, respectively.

Table 1: Limit of detection results of H5N1, H5NX and H7 isolates obtained by real time RT-PCR assays

	Plasmid copies/reaction	Limit of detection /10 tests	Limit of detection (%)
H5N1	10^{-1}	1/10	10%
	10^{-2}	0/10	0%
H5NX	10	3/10	30%
	1	0/10	0%
H7	10	10/10	100%
	1	0/10	0%

No specimen resulted positive to H5N1. For subtype H5NX, 1/70 (1.4%) cloacal swabs and 1/14 (7.1%) tracheal swabs were positive. As regards subtype H7, 1/70 (1.4%) cloacal swabs, 3/14 (21.4%) tracheal swabs and 6/12 (50%) organ pools from domestic birds resulted positive. No organ pool from wild birds resulted positive.

Discussion and conclusions

Currently there is no real time RT-PCR assay that is able to detect all H5 and H7 strains isolated so far. In our study, we have developed specific and sensitive real time RT-PCR assays that can detect all H5 and H7 subtypes worldwide since 2000 and exhibit several advantages. To obtain this, we have inevitably used degenerate primer and probe sets, with different optimized concentrations, leading to an asymmetric PCR in order to privilege the more degenerated primer.

The comparison of real time RT-PCR assays with conventional VI showed that our molecular assays were more sensitive, in agreement with their potential to detect non-infectious particles, present in the largest percentage of some virus preparations. Therefore, the real time RT-PCR assays could be used as an useful tool for rapid detection and screening of H5 and H7 isolates during influenza A virus outbreaks. Additionally, real time RT-PCR assays are less expensive than VI and results are available much faster. In conclusion, we have developed molecular assays as the most suitable alternative, in terms of time, costs and sensitivity, to conventional VI in embryonating chicken eggs for detection of H5 and H7 subtypes of AI viruses.

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DETECTION OF BLUETONGUE SEROTYPE 4 IN WAX-EMBEDDED TISSUES FROM WILD RUMINANTS IN THE SOUTH OF SPAIN

Belén RODRÍGUEZ-SÁNCHEZ[1], Pedro J. SÁNCHEZ-CORDÓN[2], Verónica MOLINA[2], María A. RISALDE[2], Ana Cristina Pérez DE DIEGO[1], José C. GÓMEZ-VILLAMANDOS[2], José M. SÁNCHEZ-VIZCAÍNO[1]

[1]Animal Health Department, Veterinary Faculty, Universidad Complutense de Madrid, Spain
[2]Deparment of Comparative Pathology, Veterinary Faculty, University of Córdoba, Spain

Keywords: bluetongue, mouflons, RT-PCR, wax-embedded tissues

Introduction and objectives

Bluetongue (BT) is a noncontagious, vector-borne disease of domestic and wild ruminants caused by an Orbivirus (Family Reoviridae) that includes at least 24 different serotypes (1, 2). Wild ruminants have been considered for a long time as asymptomatic hosts. However, recent studies have reported BT characteristic symptoms (3) and the presence of the virus (4) or BT specific antibodies (5,6) in wild species like yaks (*Bos grunniens grunniens*), mouflons (*Ovis aries musimon*) or wild red deer (*Cervus elaphus*), respectively. The role of wildlife in the prevalence and spreading of BT is beginning to be elucidated (6). The analysis of samples from wild ruminants is becoming increasingly important in order to develop control programs and to elucidate the role of wildlife in the spreading and persistence of the disease. Recently, a study on the presence of BTV antibodies in wild ruminants in Spain (6) and the characterization of BTV-1 in mouflons (*Ovis aries musimon*) (4) have been published. The adaptation of rapid molecular techniques, such as real-time PCR, to these samples can provide valuable information about the BTV serotype present in them, just by sequencing the PCR product. The advantages provided by the PCR technique –rapidity, high through-put, robustness- could replace or accompany seroneutralization as a method to characterize BTV serotypes in wildlife, provided that PCR tests developed for BTV are standardized for wildlife species.

In October 2007, two mouflons (*Ovis aries musimon*) -one male and one female- were found dead in the North of Córdoba province (South of Spain). The animals were referred to the Diagnostic Services of the Veterinary Faculty (Córdoba University) for routine necropsy. All the lesions described were compatible with BT. One year after the death of the mouflons, wax-embedded tissue samples from both individuals were included in a retrospective study in order to 1) determine the serotype of BT that caused the death of the mouflons and 2) standardize the laboratory conditions for the detection of BTV serotypes by RT-PCR from wax-embedded samples.

Materials and methods

Wax-embedded samples remaining after the study carried out by the Diagnostic Services of the Veterinary Faculty (Córdoba University, Spain) and were kept at RT. One year after they were processed, the slides were included in a retrospective study that tentatively tried to identify the BTV serotype that caused the death of the mouflons.

After trimming the excess of paraffin, tissues were scrapped off from the slides and their RNA was extracted using RNeasy® FFPE kit (Qiagen) following the manufacturer's instructions. RNA was resuspended in 20µl of PCR-quality water and frozen at -80ºC. 3µl of the RNA obtained were amplified by RT-PCR using three sets of primers that 1) amplify a common sequence in all the serotypes, within the segment 5 (7), 2) flank a 765bp sequence within the segment 2 of BTV-1 (8) and 3) overlap a unique sequence of nucleotides within BTV segment 2, that is only present in BTV serotype 4 (9). Therefore, the detection of BTV in the analyzed samples was carried out by using a gel-based, group-specific RT-PCR assay and two serotype-specific RT-PCR tests for BTV-1 and BTV-4, the serotypes present in Spain in 2007 (Rodriguez-Sanchez et al., 2008). The assay for BTV-1 is a gel-based RT-PCR and the one for BTV-4 is carried out by real-time RT-PCR. RNA extracted from purified BTV-4 (SPA-1/2004) and BTV-1 (ALG2006/01) was used as positive controls. The experiment was carried out blindly, using a code to identify each sample.

Results

Two samples from lung tissue and lymph node, one from each mouflon, gave a positive plot for BTV-4 real-time RT-PCR. The RT-PCR product -124bp- was purified with QIAquick PCR Purification Kit (Qiagen) and sequenced in an ABI 377 DNA sequencer. When the sequence from the positive samples was compared with other sequences from GenBank using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the homology with other BTV-4 segment 2 sequences ranged between 90% and 100%.

Discussion and conclusions

To the authors' knowledge, this is the first identification of BTV-4 in wild ruminants. The fact that the analysed samples from which BTV-4 was detected had been fixed and paraffin-embedded for over one year prior to the isolation of BTV RNA proves that this method preserves tissue samples without destroying the nucleic acids, thus, allowing the detection of putative pathogens present in them. Therefore, the implementation of specific RT-PCR tests to fixed samples facilitates the development of retrospective studies. This methodology provides an efficient and rapid tool for surveillance and epidemiological studies.

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GENOME SEQUENCE OF A BORDER DISEASE VIRUS STRAIN ISOLATED FROM A PYRENEAN CHAMOIS

Stefan VILCEK[1], Kim WILLOUGHBY[2], Peter F. NETTLETON[2], Peter BECHER[3]

[1]*University of Veterinary Medicine, Kosice, Slovakia;*
[2]*Moredun Research Institute, Pentlands Park, Penicuik, Midlothian EH26 0PZ, UK;*
[3]*University of Veterinary Medicine, D 30559 Hannover, Germany*

Key words: border disease virus, chamois, pestivirus

Introduction and objectives

Our knowledge of pestivirus infections in wild animals is very limited. Pestiviruses have been isolated for example from giraffe, deer, bison, bongo, reindeer, pronghorn and other animals. While some pestivirus isolates were typed at the genetic level as Bovine viral diarrhea virus 1 (BVDV-1), others were typed as Border disease virus (BDV) (reindeer) or formed separate phylogenetic branches (giraffe, pronghorn) (5).

Recently it was observed that in the Pyrenean chamois a virus was circulating which could be associated with death and a decrease in the population of these wild animals (4). A pestivirus isolated from dead animals was typed as BDV genotype 4 (1).

The aim of this work was to sequence the entire genome of this BDV isolate to obtain information on the organization of its genome and to allow comparison with other pestivirus genomes.

Material and methods

Total RNA of a virus stock (H2121) was extracted using the Viral RNA minikit (Qiagen). The cDNA was prepared using random hexamers and Moloney Murine Leukaemia Virus reverse transcriptase. Different PCR products of the pestivirus genome for sequencing were prepared using single or nested PCR employing panpestivirus or chamois virus specific primers and proofreading DNA polymerase. Longer fragments were prepared by the Expand Long Template system (Roche).

For determination of the 5´ and 3´ terminal sequences, an RNA ligation method was employed as described (3). In brief, total RNA from infected cells was ligated by using T4 RNA ligase as recommended by the supplier. Then single RT-PCR with oligonucleotides OL 380R and OL H2121-12220 was carried out. A second, nested PCR, was performed with oligonucleotides OL H2121-12250 and OL 200R (2, 3).

Most nucleotide sequences were proofread using the SeqMan II program from DNASTAR (Lasergene). The percentage of nucleotide and deduced amino acid similarities between chamois virus and selected pestivirus sequences from GenBank were calculated using the MegAlign program. Phylogenetic analysis was carried out using Neighbor-joining method (program NEIGBOR) from the phylogeny inference package programs PHYLIP.

Results

The entire genome of a pestivirus H2121 isolated from chamois is 12.305 nucleotides long. The open reading frame (ORF) is flanked with 376 nt long 5´-UTR and 232 nt long 3´-UTR. The analysis of ORF, which encodes a polyprotein consisting of 3.895 amino acids (aa), revealed that there are 490 strongly basic aa (K, R), 435 strongly acidic aa (D, E), 1.342 hydrophobic aa (A, I, L, F, W, V) and 1005 polar aa (N, C, Q, S, T, Y). The molecular weight for hypothetical polyprotein is 437.795 kDa with calculated pI = 8.527. All proteolytically cleaved viral proteins are identical or similar in size as for BDV strain X818 (ref. 5, GenBank No NC003679), e.g. N^{pro} = 168 aa, C = 100 aa, E^{rns} = 227 aa, E1 = 195 aa, E2 = 373 aa, p7 = 70 aa, NS4A = 64 aa, NS4B = 347 aa, NS5A = 497 aa, NS5B = 717 aa. However, NS2-3 region, where no significant insertions/deletions were found, is 1140 aa long, while 1136 aa were found in strain X818. Some chamois pestivirus clones contained E2 with 372 aa (due to GCT deletion corresponding to amino acid alanin).

Percentage of the amino acid similarity between BDV strain X818 (BDV-1), reindeer (BDV-2) and chamois strain (BDV-4) revealed that the highest values (over 90 %) were observed in E^{rns}, NS3, NS4A and NS4B regions, while in other regions values varied in a range of 74 to 85%. When chamois pestivirus genome was compared with the genomes from other pestivirus species, the closest values were found to CSFV (strain Alfort-T), than for BVDV-1 (SD1), BVDV-2 (890) and giraffe strain (H138). For example, for E^{rns}, E2, NS3 and NS5B corresponding values for BDV isolates (X818, reindeer) were 92-94%, 76-79%, 96-98% and 83-86%, respectively. The calculated values for other pestiviruses were as follows: 85, 68, 93 and 79% for CSFV, 78, 63, 91 and 73 for BVDV-1, 78, 58, 90 and 71% for BVDV-2 and 81, 59, 91 and 74% for giraffe strain.

Phylogenetic analysis carried out of entire genomic sequence placed chamois pestivirus into BDV branch. The analysis carried out in N^{pro} and E2 regions, for which are more sequences available in GenBank, again confirmed that chamois pestivirus belongs to BDV-4 genotype.

Discussion and conclusions

All parameters tested for the chamois pestivirus genome revealed that this virus is the most similar to border disease virus. Similar to other BDV strains, it is closer to CSFV than to other pestivirus species. The above mentioned observation was also confirmed by phylogenetic analysis. The chamois pestivirus strain is the forth BDV strain (X818, BD31, reindeer strain, chamois strain) for which the entire genome sequence is available.

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SEARCH FOR HEPATITIS E VIRUS IN WILD LIFE RESERVOIRS AND CHARACTERIZATION OF THE VIRUS IN SWEDISH WILD BOARS BY FULL LENGTH SEQUENCING

Frederik WIDÉN[1], Anna MATYI-TOTH[1], Heléne NORDER[2], Sándor BELÁK[1]

[1]Department of Virology Immunobiology and Parasitology (VIP), Statens Veterinärmedicinska Anstalt (SVA), Uppsala, Sweden;
[2]Department of enterovirus and hepatitis virus, Swedish Institute for disease control (SMI), Solna, Sweden

Keywords: hepatitis E virus, HEV, wild boar

Introduction and objectives
Hepatitis E virus (HEV) is a small RNA virus with capability to infect several animal species including humans. It has been proven that humans can be infected with HEV genotype 3 or 4 by consuming undercooked meat or liver from wild boar, pig and deer. Wild boars are present in south Sweden and stable populations are present in areas of high pig density. Previous studies have shown that Hepatitis E virus is present in Swedish pigs and wild boars. Furthermore it has been demonstrated that HEV detected in wild boars in Sweden show some similarity to HEV detected in pigs and humans. This indicates that a link between infection in wild boars, pigs and humans exists but it is not known whether this similarity is due to recent transfer of virus between these species. Furthermore it is not known if other Swedish animal species like for example Roe Deer can are infected with HEV and constitute a reservoir for infection. It is of international interest to gather such information since this has only been studied briefly and it is not known how humans acquire infection with HEV genotype 3.

Materials and methods
Blood samples and organ samples from wild boars and organ samples from Roe Deers were collected in South Sweden and analysed by PCR for presence of HEV. Positive samples were sequenced in ORF1 and ORF2 in order to determine genotype and sub group. The complete genome of selected HEV positive samples was sequenced. Sequences were compared by phylogeny.

Results
The prevalence of HEV in pig dense areas was studied. The relationship between HEV isolate from wild boars, pigs and humans was analyzed. Results indicate a close relationship between strains in wild boars, pigs and humans. Result regarding phylogenetic relationship of full length and partial sequences form HEV detected in wild boar, pigs and humans will be presented. Furthermore results of the survey for HEV in Roe deer will be presented.

Discussion and conclusions
It is evident that a link between infection in humans, wild boars and pigs may exist in the studied areas. However it is not known when the transfer of virus took place. This may be a historical event or something that are occurring at present. The results of our study provide new insight into an area that is of crucial importance for understanding the zoonotic potential of HEV.

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Author Index - Alphabetical list

List of participants and e-mail addresses

Ackermann	Mathias	Switzerland	mathias.ackermann@vetvir.unizh.ch
Aebischer	Andrea	Switzerland	andrea.aebischer@ivi.admin.ch
Akashi	Hiroomi	Japan	akashih@mail.ecc.u-tokyo.ac.jp
Albayrak	Harun	Turkey	harunalbayrak55@msn.com
Alkan	Feray	Turkey	falkan@veterinary.ankara.edu.tr
Alyabis	Adel Salih	Iraq	alsaffar51@yahoo.com
Arias	Maria Luisa	Spain	arias@inia.es
Baborenko	Elena Pavlovna	Russia	stakhanova@arriah.ru
Bächlein	Christine	Germany	christine.baechlein@tiho-hannover.de
Bahuon	Céline	France	cbahuon@vet-alfort.fr
Bakonyi	Tamás	Hungary	bakonyi.tamas@aotk.szie.hu
Balatinec	Jelena	Croatia	balatinec@veinst.hr
Bálint	Ádám	Sweden	adam.balint@bvf.slu.se
Balka	Gyula	Hungary	balka.gyula@aotk.szie.hu
Ballmann	Mónika	Hungary	ballmann@vmri.hu
Barbic	Ljubo	Croatia	ljubo.barbic@vef.hr
Bartak	Pavel	Czech Republic	bartak@svujihlava.cz
Basic	Ana	Austria	ana.basic@vu-wien.ac.at
Bassem	Salem	Egypt	mmmh22003@yahoo.com
Baule	Claudia	Sweden	claudia.baule@bvf.slu.se
Becher	Paul	Germany	paul.becher@tiho-hannover.de
Belák	Katinka	Sweden	katinka.belak@sva.se
Belák	Sándor	Sweden	sandor.belak@sva.se
Benetka	Viviane	Austria	viviane.benetka@vu-wien.ac.at
Benkő	Mária	Hungary	benko@vmri.hu
Benyeda	Zsófia	Hungary	benyeda.zsofia@aotk.szie.hu
Berg	Mikael	Sweden	mikael.berg@bvf.slu.se
Bertagnoli	Stéphane	France	s.bertagnoli@envt.fr
Bidokhti	Mehdi	Sweden	mehdi.bidokhti@sva.se
Bilge-Dagalp	Seval	Turkey	sevalbilge@hotmail.com
Billinis	Charalambos	Greece	billinis@vet.uth.gr
Blahak	Silvia	Germany	silvia.blahak@cvua-owl.nrw.de
Blomström	Anne-Lie	Sweden	anne-lie.blomstrom@bvf.slu.se
Bognár	Gábor	Hungary	gabor.bognar@sanofi-aventis.com
Bohm	Maren	Germany	maren.bohm@tiho-hannover.de
Borchers	Kerstin	Germany	borchers@zedat.fu-berlin.de
Borrego	Belen	Spain	borrego@inia.es
Bouet-Cararo	Coraline	France	cbouet@vet-alfort.fr
Breum	Solvej O.	Denmark	sbre@vet.dtu.dk
Brown	Ian H.	UK	i.h.brown@vla.defra.gsi.gov.uk
Brunet	Sylvie	France	sylvie.brunet@merial.com
Bublot	Michel	France	michel.bublot@merial.com
Buonavoglia	Canio	Italy	c.buonavoglia@veterinaria.uniba.it
Calvo Pinilla	Eva	Spain	cpinilla@inia.es
Cardeti	Giusy	Italy	Giusy.cardeti@izslt.it
Chacon Villanueva	Jorge Luis	Hungary	Jorge.chacon@ceva.com
Clegg	Simon R.	UK	s.r.clegg@liv.ac.uk

Colaianni	Maria Loredana	Italy	lorivet@libero.it
Comtet	Loic	France	loic.comtet@id-vet.com
Cordonnier	Nathalie	France	ncordonnier@vet-alfort.fr
Crooke	Helen R.	UK	h.crooke@vla.defra.gsi.gov.uk
Czifra	Georg	Ireland	georg.czifra@ec.europa.eu
Csire	Márta	Hungary	csire.marta@oek.antsz.hu
Dal Pozzo	Fabiana	Belgium	fdalpozzo@ulg.ac.be
Dán	Ádám	Hungary	dana@oai.hu
Dandár	Eszter	Hungary	dandareszti@yahoo.co.uk
Dastjerdi	Akbar	UK	a.dastjerdi@vla.defra.gsi.gov.uk
De Benedictis	Paola	Italy	pdebenedictis@izsvenezie.it
De Leeuw	Ilse	Belgium	Ilse.deleeuw@var.fgov.be
De Mia	Gian Mario	Italy	gm.demia@izsum.it
de Smit	A. J. (Hans)	Netherlands	hans.desmit@sp.intervet.com
De Vriese	Joëlle	Belgium	jodev@var.fgov.be
Decaro	Nicola	Italy	n.decaro@veterinaria.uniba.it
Dedet	Vincent G. D.	France	vdedet@invivo.edu
Demeter	Zoltán	Hungary	demeter.zoltan@aotk.szie.hu
Diederichs	Meike	Germany	meike.diederichs@tiho-hannover.de
Dirbakova	Zuzana	Slovakia	dirbakova@svuzv.sk
Doherty	Simon	UK	simon.doherty@afbini.gov.uk
Dosedlova	Vilma	Czech Republic	dosedlova@uskvbl.cz
Doszpoly	Andor	Hungary	adoszpoly@vmri.hu
Drén	Csaba	Hungary	drencsaba@gmail.com
Duffy	Kate	UK	c.duffy@qub.ac.uk
Duthie	Susan	UK	susan.duthie@biobest.co.uk
Edwards	Steven	UK	vet@cabanas.waitrose.com
El Saied	Mohamed	Egypt	hamed.yosef@yahoo.com
Elizalde	Maia	Spain	melizald@tragsa.es
Eloit	Marc	France	meloit@vet-alfort.fr
Emmoth	Eva	Sweden	eva.emmoth@sva.se
Erdélyi	Károly	Hungary	kerdelyi@gmail.com
Espunya	Enric	Spain	eem@hipra.com
Eymann-Haeni	Rita	Switzerland	rita.eymann-haeni@ivi.admin.ch
Farkas	Tibor	Hungary	tibor.farkas@ceva.com
Farsang	Attila	Hungary	farsanga@oai.hu
Feliziani	Francesco	Italy	f.feliziani@izsum.it
Ferenczi	Emőke G.	Hungary	ferenczi.emoke@oek.antsz.hu
Fernández-Pinero	Jovita	Spain	fpinero@inia.es
Field	Hugh J.	UK	hjf10@cam.ac.uk
Foni	Emanuela	Italy	emanuela.foni@izsler.it
Forgách	Petra	Hungary	forgach.petra@aotk.szie.hu
Forgách	Katalin	Hungary	katalin.forgach@ceva.com
Förster	Christine	Germany	christine.foerster@vetmed.uni-giessen.de
Fragkiadaki	Eftychia	Greece	efxil@aua.gr, eftychia.xylouri@yahoo.gr
Friedgut	Orly	Israel	orlyf@moag.gov.il
Fritsche	Ann-Kathrin	Germany	akfritsche@gmx.de
Gadd	Tuija	Finland	tuija.gadd@evira.fi
Gál	János	Hungary	gal.janos@aotk.szie.hu
Galligioni	Viola	Italy	violagialla@yahoo.it
Garcon	Fanny	UK	f.garcon@vla.defra.gsi.gov.uk
Gilgenbach	Stefanie	Germany	stefanie.gilgenbach@tiho-hannover.de
Graham	David A.	UK	david.graham@afbini.gov.uk
Gransden	Dora	UK	dora.gransden@qiagen.com
Grasland	Beatrice S. M.	France	b.grasland@ploufragan.afssa.fr
Gretillat	Magalie	France	m.gretillat@envt.fr
Grierson	Sylvia S.	UK	s.grierson@vla.defra.gsi.gov.uk

Grom	Joze	Slovenia	joze.grom@vf.uni-lj.si
Grummer	Beatrice	Germany	beatrice.grummer@tiho-hannover.de
Guelbenzu	Maria P.	UK	maria.guelbenzu@afbini.gov.uk
Gyuranecz	Miklós	Hungary	m.gyuranecz@gmail.com
Ha	Hye Jeong	New Zealand	h.j.ha@massey.ac.nz
Hakhverdyan	Mikhayil	Sweden	mikhayil.hakhverdyan@sva.se
Hannant	Duncan	UK	duncan.hannant@nottingham.ac.uk
Hanoun	Yakoub Fargalla	Egypt	hamed.yosef@yahoo.com
Harmeyer	Silke	UK	silke.harmeyer@pfizer.com
Harrach	Balázs	Hungary	harrach@vmri.hu
Henrych	Denise	Germany	denise.henrych@tiho-hannover.de
Herrler	Georg	Germany	georg.herrler@tiho-hannover.de
Hjulsager	Charlotte Kristiane	Denmark	ckhj@vet.dtu.dk
Hofmann	Martin	Switzerland	martin.hofmann@ivi.admin.ch
Hornyák	Ákos	Hungary	hornyak.akos@aotk.szie.hu
Horsington	Jacquelyn J.	Australia	jacquelynjh@gmail.com
Horzinek	Marian C.	Netherlands	horzinek@gmail.com
Jánoska	Máté	Hungary	janoskam@vmri.hu
Jensen	Trine H.	Denmark	trje@vet.dtu.dk
Jestin	André	France	a.jestin@afssa.fr
Jestin	Véronique	France	v.jestin@afssa.fr
Juhász	Tamás	Hungary	juhaszta@oai.hu
Kaján	Győző	Hungary	gykajan@vmri.hu
Kériné Balogh	Zsuzsanna	Hungary	balogh.zsuzsanna@oek.antsz.hu
Kirisawa	Rikio	Japan	r-kirisa@rakuno.ac.jp
Kiss	István	Sweden	istvan.kiss@sva.se
Klein	Dieter	Austria	dieter.klein@vu-wien.ac.at
Klewer	Kristine	France	kristine.klewer@id-vet.com
Koenen	Frank	Belgium	frkoe@var.fgov.be
Korpenfelt	Sirkka-Liisa	Finland	sirkka-liisa.korpenfelt@evira.fi
Kovács	Endre R.	Hungary	ekovacs@vmri.hu
Kökény	Szabolcs	Hungary	Szabolcs.Kokeny@lifetech.com
König	Patricia	Germany	patricia.koenig@fli.bund.de
Kővágó	Csaba	Hungary	Kovago.Csaba@aotk.szie.hu
Krell	Peter J.	Canada	pkrell@uoguelph.ca
Kristersson	Therese	Sweden	therese.kristersson@svanova.com
Kuehn	Katrin	Germany	katrin.kuehn@fli.bund.de
Kukielka	Deborah	Spain	deborah@sanidadanimal.info
Kukushkin	Sergey A.	Russia	sergey.kukushkin@mail.ru
Kulcsár	Gábor	Hungary	kulcsarg@oai.hu
Kuntz-Simon	Gaëlle	France	g.kuntz.simon@afssa.fr
La Rocca	S. Anna	UK	a.larocca@vla.defra.gsi.gov.uk
Lange	Anastasia	Germany	anastasia.lange@tiho-hannover.de
Le Gall-Reculé	Ghislaine	France	g.legall@afssa.fr
Le Poder	Sophie	France	slepoder@vet-alfort.fr
Lea	Richard G.	UK	richard.lea@nottingham.ac.uk
Lecollinet	Sylvie	France	s.lecollinet@afssa.fr
Leifer	Immanuel	Germany	Immanuel.Leifer@fli.bund.de
Lesbros	Cynthia	France	cynthia.lesbros@virbac.com
Letellier	Carine	Belgium	calet@var.fgov.be
Li	Zhiyong	China	li_zhiyong56@yahoo.com.cn
Lindhe	Anna	Sweden	anna.lindhe@sva.se
Lipkin	W. Ian	USA	wil2001@columbia.edu
Liu	Lihong	Sweden	lihong.liu@sva.se
López-Olvera	Jorge R.	Spain	jordi.lopez.olvera@uab.es
Lőrincz	Márta	Hungary	lorincz.marta@aotk.szie.hu
Luk	So-mui, Geraldine	China	loretta_wc_lee@afcd.gov.hk

Maclachlan	N. James	USA	njmaclachlan@ucdavis.edu
Madić	Josip	Croatia	josip.madic@vef.hr
Maes	Roger K.	USA	maes@dcpah.msu.edu
Mahapatra	Mana	UK	mana.mahapatra@bbsrc.ac.uk
Malik	Péter	Hungary	pmalik@freemail.hu
Mandola	Maria Lucia	Italy	virologia.ovocoltura@izsto.it
Mari	Viviana	Italy	v.mari@veterinaria.uniba.it
Mars	Maria H.	Netherlands	m.mars@gddeventer.com
Marschang	Rachel E.	Germany	rachel.marschang@googlemail.com
Martella	Vito	Italy	v.martella@veterinaria.uniba.it
Martelli	Francesca	UK	f.martelli@vla.defra.gsi.gov.uk
Martinelle	Ludovic	Belgium	lmartinelle@ulg.ac.be
Martins	Carlos L.V.	Portugal	cmartins@fmv.utl.pt
Masoero	Loretta	Italy	loretta.masoero@izsto.it
Mathijs	Elisabeth	Belgium	emathijs@ulg.ac.be
Mató	Tamás	Hungary	tamas.mato@ceva.com
Mauroy	Axel	Belgium	amauroy@ulg.ac.be
McKillen	John	UK	John.McKillen@afbini.gov.uk
McMenamy	Michael J.	UK	m.mcmenamy@qub.ac.uk
McNab	Tegan J.	Australia	tegan.mcnab@gmail.com
Metreveli	Giorgi	Sweden	giorgi.metreveli@sva.se
Mettenleiter	Thomas C.	Germany	thomas.mettenleiter@fli.bund.de
Meyer	Gilles	France	g.meyer@envt.fr
Minguez-Tudela	Isabel	Belgium	Isabel.minguez-tudela@ec.europa.eu
Moennig	Volker	Germany	volker.moennig@tiho-hannover.de
Mojzis	Miroslav	Slovakia	mojzis@svuzv.sk
Monne	Isabella	Italy	imonne@izsvenezie.it
Montero Calle	Jana Laia	Spain	mcalle@inia.es
Moulin	Véronique	Netherlands	veronique.moulin@sp.intervet.com
Muller	Alexandra	Portugal	amuller@mail.icav.up.pt
Muñoz Reoyo	María J.	Spain	reoyo@inia.es
Must	Külli	Estonia	kylli.must@vetlab.ee
Nagy	Béla	Hungary	bnagy@vmri.hu
Nagy	Éva	Canada	enagy@uoguelph.ca
Nájera Lomelí	Wilfrido	Mexico	wnajera@gmail.com
Nardelli	Stefano	Italy	snardelli@izsvenezie.it
Newsham	Emma	UK	emma.newsham@liv.ac.uk
Nilsson	Ernst	Sweden	ernst.nilsson@svanova.com
Nollens	Hendrik	USA	nollensh@vetmed.ufl.edu
Nordengrahn	Ann	Sweden	ann.nordengrahn@svanova.com
Nowotny	Norbert	Austria	norbertnowotny@gmx.at
Ntafis	Vasileios	Greece	vasilisntafis@hotmail.com
Oliveira-Filho	Edmilson F.	Germany	edmilsonz@yahoo.com.br
Ortego	Javier	Spain	ortego@inia.es
Oura	Chris A. L.	UK	chris.oura@bbsrc.ac.uk
Palade	Elena Alina	Hungary	palade.alina@aotk.szie.hu
Palya	Vilmos	Hungary	vilmos.palya@ceva.com
Pannhorst	Katrin	Germany	katrin.pannhorst@tiho-hannover.de
Pantó	Laura	Hungary	panto@vmri.hu
Papp	Tibor	Germany	kabafalvi@gmail.com
Pastoret	Paul-Pierre	France	pp.pastoret@oie.int
Pénzes	Judit	Hungary	penzes@vmri.hu
Pérez de Diego	Ana C.	Spain	anacristina@sanidadanimal.info
Perez-Sancho	Marta	Spain	maperezs@visavet.ucm.es
Petrovszki	Enikő	Hungary	eniko.petrovszki@ceva.com
Pezzoni	Giulia	Italy	giulia.pezzoni@izsler.it
Pitti	Monica	Italy	monica.pitti@unimi.it

Pizzuto	Matteo S.	Italy	mpizzuto@izsvenezie.it
Pourquier	Philippe	France	philippe.pourquier@id-vet.com
Punyadarsaniya	Darsaniya	Germany	darsaniya.punyadarsaniya@tiho-hannover.de
Rabalski	Lukasz	Poland	lukaszr001@gmail.com
Radford	Alan D.	UK	alanrad@liv.ac.uk
Reimann	Ilona	Germany	ilona.reimann@fli.bund.de
Rigoni	Michela	Italy	mrigoni@izsvenezie.it
Rodríguez-Sánchez	Belén	Spain	mbrodrig@vet.ucm.es
Rosado	Ricardo C.	Portugal	rcrosado@gmail.com
Rottier	Peter J. M.	Netherlands	p.rottier@uu.nl
Römer-Oberdörfer	Angela	Germany	angela.roemer-oberdoerfer@fli.bund.de
Rusvai	Miklós	Hungary	rusvai.miklos@aotk.szie.hu
Sala	Marcello G.	Italy	marcello.sala@izslt.it
Sánchez-Vizcaíno	José Manuel	Spain	jmvizcaino@visavet.ucm.es
Schelp	Christian	Switzerland	christian-schelp@idexx.com
Schlegel	Michael	Germany	michael.schlegel@idt-biologika.de
Schmeiser	Stefanie	Germany	stefanie.deike@tiho-hannover.de
Schmidt	Ulrike	Netherlands	ulrike.schmidt@sp.intervet.com
Schreiber	Paul	France	paul.schreiber@virbac.com
Scicluna	Maria Teresa	Italy	teresa.scicluna@izslt.it
Selbitz	Hans-Joachim	Germany	hans-joachim.selbitz@idt-biologika.de
Sihvonen	Liisa Helena	Finland	liisa.sihvonen@evira.fi
Skoda	Gabriella	Hungary	skoda_gabriella@freemail.hu
Smitalova	Radka	Czech Republic	smitalova@uskvbl.cz
Smyth	Victoria J.	UK	v.j.smyth@qub.ac.uk
Snoeck	Chantal J.	Luxembourg	chantal.snoeck@lns.etat.lu
Spyrou	Vassiliki	Greece	spyrou@teilar.gr
Stipkovits	László	Hungary	stipkovits@vmri.hu
Szatmáry	Susan	USA	
Takács	Mária	Hungary	takmar@gmail.com
Tarján	László Zoltán	Hungary	lztarjan@gmail.com
Tatár-Kis	Tímea	Hungary	timea.tatar@ceva.com
Thibault	Jean-Christophe	France	jean-christophe.thibault@merial.com
Thiery	Richard	France	r.thiery@afssa.fr
Thiry	Etienne L.	Belgium	etienne.thiry@ulg.ac.be
Tignon	Marylène	Belgium	marylene.tignon@var.fgov.be
Todd	Daniel	UK	daniel.todd@afbini.gov.uk
Top	Sokunthea	France	s.top@envt.fr
Toplak	Ivan	Slovenia	ivan.toplak@vf.uni-lj.si
Troeng	Jan	Sweden	jan.troeng@svanova.com
Tuboly	Tamás	Hungary	tuboly.tamas@aotk.szie.hu
Tuboly	Sándor	Hungary	tuboly.sandor@aotk.szie.hu
Tuppurainen	Eeva S. M.	UK	eeva.tuppurainen@bbsrc.ac.uk
Ursu	Krisztina	Hungary	ursu.krisztina@gmail.com
Vahlenkamp	Thomas W.	Germany	thomas.vahlenkamp@fli.bund.de
van de Wetering	Gerard	Netherlands	gerard.vandewetering@prionics.com
van den Berg	Thierry P. R. P.	Belgium	thvan@var.fgov.be
van der Poel	Wim H. M.	Netherlands	wim.vanderpoel@wur.nl
van Maanen	Kees	Netherlands	c.v.maanen@gddeventer.com
Van Reeth	Kristien	Belgium	kristien.vanreeth@ugent.be
van Rijn	Piet A.	Netherlands	piet.vanrijn@wur.nl
Vanbinst	Tine	Belgium	tine.vanbinst@var.fgov.be
Venter	Estelle H.	South Africa	estelle.venter@up.ac.za
Vidovszky	Márton	Hungary	vidovszky@vmri.hu
Vilcek	Stefan	Slovakia	vilcek@uvm.sk
Villafuerte Munoz	Ricardo	Mexico	ric_villafuerte@yahoo.com.mx
Volmer	Romain	France	r.volmer@envt.fr

Walk	Karin	Austria	karin.walk@vu-wien.ac.at
Watanabe	Shumpei	Japan	aa67157@mail.ecc.u-tokyo.ac.jp
Weissenböck	Herbert	Austria	herbert.weissenboeck@vu-wien.ac.at
Wellehan	James	USA	wellehanj@vetmed.ufl.edu
Wensman	Jonas J.	Sweden	Jonas.Wensman@bvf.slu.se
Widén	Frederik	Sweden	frederik.widen@sva.se
Wilcox	Graham E.	Australia	g.wilcox@murdoch.edu.au
Wood	John S.	Greece	qefii@gmail.com
Xia	Hongyan	Sweden	hongyan.xia@bvf.slu.se
Yacoub	Alia	Sweden	alia.yacoub@sva.se
Yin	Xiangping	China	yinxiangping115@yahoo.com.cn
Yon	Lisa K.	UK	lisa.yon@nottingham.ac.uk
Zádori	Zoltán	Hungary	zadori@vmri.hu
Zhang	Shucheng	USA	shucheng.zhang@Pfizer.com
Zientara	Stéphan	France	s.zientara@afssa.fr
Zsák	László	USA	laszlo.zsak@ars.usda.gov

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