Friedrich-Loeffler-Institut (FLI),
Institute of Farm Animal Genetics,
Neustadt-Mariensee, Germany

Comparative molecular analysis of methicillin-resistant isolates of *Staphylococcus aureus* and coagulase-negative *Staphylococcus* spp. from cases of mastitis among dairy cattle

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Andrea Theresia Feßler
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Supervisor:	Prof. Dr. Stefan Schwarz
Supervision Group:	Prof. Dr. Stefan Schwarz Prof. Dr. Günter Klein Prof. Dr. Iris F. Chaberny
1st Evaluation:	Prof. Dr. Stefan Schwarz Friedrich-Loeffler-Institut (FLI), Institute of Farm Animal Genetics, Neustadt-Mariensee Prof. Dr. Günter Klein Institute of Food Quality and Food Safety, University of Veterinary Medicine Hannover, Hannover Prof. Dr. Iris F. Chaberny Division of Hospital Epidemiology and Infection Control, Institute for Medical Microbiology and Hospital Epidemiology, Hannover Medical School, Hannover
2nd Evaluation:	Prof. Dr. Jaap A. Wagenaar Faculty of Veterinary Medicine (FVM), Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, The Netherlands
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Chapter 1

Introduction

1.1 Methicillin-resistant staphylococci as commensals and pathogens of animals

Staphylococci are Gram-positive, catalase-positive, facultatively anaerobic and immotile cocci which grow in characteristic clusters (Selbitz, 2002). The genus *Staphylococcus* consists currently of 46 recognized species and 24 subspecies (http://www.bacterio.cict.fr/s/staphylococcus.html). Based on their ability to produce coagulase, staphylococci are differentiated into two large groups, the coagulase-positive and the coagulase-negative staphylococci. Solely *Staphylococcus* (*S.*) *hyicus* is classified as coagulase-variable (Aarestrup and Schwarz, 2006). Staphylococci are common inhabitants of the skin and the mucosal surfaces of humans and various animals. As such, they constitute part of the physiological flora (Werckenthin et al., 2001; Aarestrup and Schwarz, 2006). Certain staphylococci predominantly occur in specific host animal species, e.g. *Staphylococcus pseudintermedius* in dogs (Fitzgerald, 2009), *Staphylococcus intermedius* in pigeons (Sasaki et al., 2007), *S. hyicus* in pigs (L'Ecuyer, 1967) or *Staphylococcus felis* in cats (Igimi et al., 1989). However, members of other staphylococcal species are also isolated from these animal hosts (Biberstein et al., 1984).

Most staphylococcal species are considered as facultatively pathogenic. Their pathogenic capacity mainly depends on whether and – if so – which virulence genes they carry and express (Selbitz, 2002). On the host side, the intact skin or mucosal surface represents a first mechanical barrier against these infectious agents. In addition, a functionally active host immune system as well as the tissue-specific commensal flora play important roles as biological barriers against pathogenic bacteria, including staphylococci (Tizard et al., 2004). Open injuries, burns, scratch and bite wounds, but also primary viral or parasitic infections, which destroy this mechanical barrier, enable staphylococci as inhabitants of the skin or the mucosa to reach deeper tissues and cause an either localized or generalized infection (Werckenthin et al., 2001). The latter happens mainly when the staphylococci disseminate via blood and can evade or suppress the host's immune system. Certain virulence factors of *Staphylococcus aureus*, in particular the Panton-Valentine

leukocidin, target and destroy components of the host immune system (Selbitz, 2002).

In veterinary medicine, staphylococci play a role in a wide range of diseases in various animal species, such as bovine mastitis (e.g. *S. aureus* and coagulase-negative staphylococci such as *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus chromogenes* etc.), canine pyoderma (*S. pseudintermedius*), exudative epidermitis in pigs (*S. hyicus*) or septicaemia in poultry (*S. aureus*) (Hummel and Witte, 1979; Werckenthin et al., 2001; Aarestrup and Schwarz, 2006; Fitzgerald, 2009; Pyörälä and Taponen, 2009). Among the *Staphylococcus*-associated diseases of food-producing animals, bovine mastitis is of major economical importance. A recent estimate stated that mastitis costs the U.S. dairy industry about $ 1.7-2 billion annually (Jones and Bailey, 2009). Besides their role as animal pathogens, staphylococci, which colonize food-producing animals, can contaminate carcasses during the slaughter process (Vanderlinde et al., 1999). This is of particular importance if these staphylococci are able to produce enterotoxins which can withstand heating and are present in food and food products even after the contaminating staphylococcal isolates have been killed (Balaban and Rasooly, 2000; Dinges et al., 2000). A number of staphylococcal enterotoxins have been confirmed to exhibit emetic activity and play a role in food poisoning (Dinges et al., 2000).

Staphylococcal infections in both, humans and animals, are commonly treated with antimicrobial agents (Aarestrup and Schwarz, 2006). Among them, β-lactam antibiotics play a major role in the control of staphylococci (Li et al., 2007). However, the efficacy of β-lactam antibiotics has been severely compromised by the occurrence and the spread of the methicillin resistance gene *mecA* in staphylococci (Deurenberg et al., 2007; Monecke et al., 2011). This gene codes for the alternative penicillin binding protein PBP2a, which shows a strongly reduced affinity to almost all β-lactam antibiotics (Entenza et al., 2002). Although methicillin-resistant staphylococci have rarely been seen in veterinary medicine until the year 2005, their numbers have distinctly increased during recent years (Voss et al., 2005; Witte et al., 2007; Nemati et al. 2008; Kadlec et al., 2009; Vanderhaeghen et al., 2010). In this regard, methicillin-resistant *S. aureus* (MRSA) of the clonal complex (CC) 398 is of

major relevance in veterinary medicine and food animal production. This MRSA type has also been referred to as “Livestock-Associated” MRSA (LA-MRSA) (Köck et al. 2010; Lindsay, 2010; Monecke et al., 2011).

As staphylococci, including MRSA, can be transferred between animals and humans either via direct contact, aerosols or the food chain, it is important to have diagnostic methods to characterize such isolates to the strain level and trace back their routes of transmission (Schwarz et al., 2003). For this, pattern-based approaches, such as pulsed-field gel electrophoresis, have traditionally been used (Schwarz et al., 2003). However, MRSA CC398 strains harbor a restriction/ modification system that prevents their DNA from being cut by the “gold-standard” enzyme SmaI (Bens et al., 2006). As a consequence, other restriction enzymes, such as ApaI or Cfr9I had to be used for PFGE of *S. aureus* (Kadlec et al., 2009; Rasschaert et al., 2009; Argudín et al., 2010; Bosch et al., 2010). Nowadays, mainly sequence-based molecular methods, like multilocus sequence typing (MLST) (Enright et al., 2000) and *spa* typing (Harmsen et al., 2003), are applied. Additional novel methods, like *dru* typing (Goering et al., 2008) and Multiple Locus Variable Number of Tandem Repeat analysis (MLVA) typing (Sabat et al., 2003; Schouls et al., 2009), have been developed during recent years. To date only limited information is available about their usefulness in the characterization and short-term epidemiological analyses of LA-MRSA. Furthermore, a *S. aureus*-specific DNA microarray, which simultaneously detects approximately 180 genes and alleles thereof, including a large number of virulence and antimicrobial resistance genes, has become commercially available (Monecke et al., 2007, Monecke et al., 2008a, Monecke et al., 2008b). The hybridization patterns obtained with this microarray corresponded closely to the clonal complexes (CCs) seen in MLST analysis. This observation was confirmed by the analysis of more than 3,000 clinical and veterinary MRSA isolates (Monecke et al., 2011).

1.2 Antimicrobial resistance of methicillin-resistant staphylococci of animal origin

According to the recommendations of the Clinical and Laboratory Standards Institute (CLSI), methicillin-resistant staphylococci should be considered as being resistant to all β-lactam antibiotics approved for veterinary medicine (CLSI, 2008). As β-lactam antibiotics are the major antimicrobial agents approved for and used in bovine mastitis (Constable et al., 2008), the diagnosis MRSA needs to be confirmed by appropriate clinical breakpoints and additional laboratory tests, such as PCR-directed detection of the *mecA* gene or tests for the detection of the PBP2a protein. The existing clinical breakpoints for oxacillin, which were adopted from human medicine, appeared to be appropriate for the identification of MRSA of animal origin. However, little is known about their ability to reliably detect methicillin-resistant coagulase-negative staphylococci (MRCoNS) from cases of bovine mastitis.

MRSA have been shown to be often resistant to a number of non-β-lactam antibiotics, such as tetracyclines, macrolides/lincosamides, trimethoprim and/or aminoglycosides. A closer look at the respective resistance genes so far identified in LA-MRSA CC398 revealed a wide variety of resistance genes (Monecke et al., 2007; Schwarz et al., 2008; Kadlec et al., 2009; Kadlec and Schwarz, 2009a; Kadlec and Schwarz, 2009b; Kehrenberg et al., 2009; Kadlec and Schwarz, 2010a; Kadlec et al., 2010; Argudin et al., 2011; Lozano et al., 2011a; Lozano et al., 2011b; Monecke et al., 2011, Schwendener and Perreten, 2011; Lozano et al., 2012; Price et al., 2012). These include genes which are known to be present in other staphylococci of human and animal origin, such as the β-lactamase gene *blaZ*, the tetracycline resistance genes *tet*(M) and *tet*(K), the macrolide/lincosamide/streptogramin B (MLS_B) resistance genes *erm*(A), *erm*(B) and *erm*(C), as well as the genes *aacA-aphD* or *aadD* coding for resistance to gentamicin/tobramycin/kanamycin or kanamycin/meomycin, respectively (Lyon and Skurray, 1987; Werckenthin et al., 2001). Other resistance genes are believed to originate from other Gram-positive bacteria, but have been acquired by staphylococci (including LA-MRSA ST398) via horizontal gene transfer. Examples of such resistance genes described in LA-MRSA

ST398 are the tetracycline resistance gene *tet*(L), which is believed to originate from *Bacillus* spp. (Hoshino et al., 1985; Noguchi et al., 1986), or the MLS_B resistance gene *erm*(T), which has been described to be present in *Streptococcus* spp., *Lactobacillus* spp. and *Enterococcus faecium* (DiPersio et al., 2008; Egervärn et al., 2009; Whitehead and Cotta, 2001). In addition, studies have also revealed the presence of novel resistance genes, which have been described for the first time in LA-MRSA ST398 isolates, such as the trimethoprim resistance gene *dfrK* (Kadlec and Schwarz, 2009a) and the ABC transporter gene *vga*(C) for lincosamide/ pleuromutilin/streptogramin A resistance (Kadlec and Schwarz, 2009b). So far, only *dfrK* has very recently been detected also in *Enterococcus* isolates (López et al., 2012).

Studies on the mobility and the localization of resistance genes in LA-MRSA ST398 isolates showed that many resistance genes were either plasmid- or transposon-borne (Kadlec and Schwarz, 2009b; Kehrenberg et al., 2009; Kadlec and Schwarz, 2010a; Kadlec and Schwarz, 2010b; Kadlec et al., 2010; Mendes et al., 2011; Schwendener and Perreten, 2011). Plasmids carrying the genes *tet*(L)-*dfrK* have been detected on structurally different plasmids, which can also harbour additional resistance genes such as *aadD*, *vga*(C) or *erm*(T). In addition, a recombination led to the replacement of the Tn*554*-associated resistance genes *erm*(A) and *spc* by the plasmid-borne gene *dfrK* and resulted in the formation of a novel and functionally active resistance transposon Tn*559* (Kadlec and Schwarz, 2010b) .

Co-localization of resistance genes in multi-resistance gene clusters or on multi-resistance plasmids enables not only the co-selection, but also the persistence of different resistance genes under the selective pressure imposed by the use of a single antimicrobial agent (Kadlec and Schwarz, 2009a, Kadlec and Schwarz, 2010a). Moreover, the analysis of the resistance genes and their location provides insight into the gene acquisition capacities of LA-MRSA CC398 isolates, their role as recipients and donors of resistance genes, and possible partners for gene exchange processes beyond the genus *Staphylococcus*.

1.3 Aims

The aims of the present PhD thesis were:

(1) to comparatively characterize LA-MRSA isolates from cases of bovine mastitis and from non-bovine LA-MRSA isolates (i.e. from poultry meat and poultry meat products) in Germany for their genetic relationships, their virulence and their resistance properties,

(2) to characterize LA-MRSA isolates from humans and animals in Dutch dairy farms,

(3) to characterize MRCoNS isolates from cases of bovine mastitis in Germany for their genetic relationships and their resistance properties with particular reference to the correct assessment of methicillin resistance,

(4) to make a proposal for interpretive criteria for cefoperazone susceptibility testing of bovine mastitis pathogens (including LA-MRSA and MRCoNS),

(5) to analyze plasmid-borne resistances to protein biosynthesis inhibitors among staphylococci with particular reference to the identification of the molecular basis of apramycin resistance and combined resistance to lincosamides, pleuromutilins and streptogramin A antibiotics,

(6) to investigate target gene mutations responsible for decreased susceptibility against fluoroquinolones among *S. aureus* isolates, and

(7) to investigate a refined Multiple Locus Variable Number of Tandem Repeat analysis (MLVA) approach for its ability to discriminate between LA-MRSA strains.

Chapter 2

Characterization of methicillin-resistant *Staphylococcus aureus* ST398 from cases of bovine mastitis

Feßler A*, Scott C*, Kadlec K, Ehricht R, Monecke S and Schwarz S

Journal of Antimicrobial Chemotherapy (2010) **65**, 619-625.
doi:10.1093/jac/dkq021

* both authors contributed equally to this study

Chapter 3

Characterization of methicillin-resistant *Staphylococcus aureus* isolates from food and food products of poultry origin in Germany

Feßler AT, Kadlec K, Hassel M, Hauschild T, Eidam C, Ehricht R, Monecke S and Schwarz S

Applied and Environmental Microbiology (2011) **77**, 7151-5157.
doi:10.1128/AEM.00561-11

Chapter 4

Characterization of methicillin-resistant *Staphylococcus aureus* CC398 obtained from humans and animals on dairy farms

Feßler AT, Olde Riekerink RGM, Rothkamp A, Kadlec K, Sampimon OC, Lam TJGM and Schwarz S

Veterinary Microbiology (2012) [Epub ahead of print]
doi:10.1016/j.vetmic.2012.05.005

Chapter 5

Identification and characterization of methicillin-resistant coagulase-negative staphylococci from bovine mastitis

Feßler AT*, Billerbeck C*, Kadlec K and Schwarz S

Journal of Antimicrobial Chemotherapy (2010) **65,** 1576-1582.
doi:10.1093/jac/dkq172

* both authors contributed equally to this study

Chapter 6

A proposal of interpretive criteria for cefoperazone applicable to bovine mastitis pathogens

Feßler AT, Kaspar H, Lindeman CJ, Stegemann MR, Peters T, Mankertz J, Watts JL and Schwarz S

Veterinary Microbiology (2012) **157**, 226-231.
doi:10.1016/j.vetmic.2011.11.032

Chapter 7

Plasmid-mediated resistance to protein biosynthesis inhibitors in staphylococci

Schwarz S, Feßler AT, Hauschild T, Kehrenberg C and Kadlec K

Annals of the New York Academy of Science (2011) **1241**: 82-103.
doi: 10.1111/j.1749-6632.2011.06275.x.

Chapter 8

Novel and uncommon antimicrobial resistance genes in livestock-associated methicillin-resistant *Staphylococcus aureus*

Kadlec K, Feßler AT, Hauschild T and Schwarz S

Clinical Microbiology and Infection (2012) [Epub ahead of print]
doi:10.1111/j.1469-0691.2012.03842.x.

Chapter 9

Novel apramycin resistance gene *apmA* in bovine and porcine methicillin-resistant *Staphylococcus aureus* ST398 isolates

Feßler AT, Kadlec K and Schwarz S

Antimicrobial Agents Chemotherapy (2011) **55**, 373-375.
doi:10.1128/AAC.01124-10

Chapter 10

Small plasmids carrying the novel resistance genes *dfrK* or *apmA* isolated from methicillin-resistant or -susceptible staphylococci

Kadlec K, Feßler AT, Pomba CF, Couto N and Schwarz S

Journal of Antimicrobial Chemotherapie
(accepted)

Unusual small plasmids carrying the novel resistance genes *dfrK* or *apmA* isolated from methicillin-resistant or -susceptible staphylococci

Kristina Kadlec [1], Andrea T. Feßler [1], Natacha Couto [2], Constança F. Pomba [2], and Stefan Schwarz [1,*]

[1] *Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, Neustadt-Mariensee, Germany;*

[2] *Interdisciplinary Centre of Research in Animal Health, Faculty of Veterinary Medicine, Technical University of Lisbon, Lisboa, Portugal*

Short title: small resistance plasmids in MRSA ST398

Keywords: MRSA, trimethoprim resistance, apramycin resistance, horizontal gene transfer

* Corresponding author: Mailing address: Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Höltystr. 10, 31535 Neustadt-Mariensee, Germany. Phone: +49-5034-871-241. Fax: +49-5034-871-143. Electronic mail address: stefan.schwarz@fli.bund.de

Synopsis

***Objectives*:** The aims of this study were to identify small staphylococcal plasmids that carry either the trimethoprim resistance gene *dfrK* or the apramycin resistance *apmA* and analyse them for their structure and organisation with regard to their potential role as precursors of large multiresistance plasmids that carry these genes.

***Methods*:** Trimethoprim- or apramycin-resistant staphylococci from the strain collections of the two participating institutions were investigated for the presence of plasmid-borne *dfrK* or *apmA* genes. The *dfrK*- or *apmA*-carrying plasmids were sequenced completely and compared to sequences deposited in the databases.

***Results*:** Two small plasmids, the 4,957 bp *dfrK*-carrying plasmid pKKS966 from porcine *Staphylococcus hyicus* subsp. *hyicus* and the 4,809 bp *apmA*-carrying plasmid pKKS49 from porcine methicillin-resistant *S. aureus* were identified. Structural analysis revealed that both plasmids had a similar organisation comprising a single resistance gene (*dfrK* or *apmA*), a plasmid replication gene (*rep*) and three in part overlapping genes for mobilization proteins (*mobA*, *mobB*, and *mobC*). Comparisons showed 71–82% amino acid identity between the Rep and Mob proteins of these two plasmids; however, distinctly lesser percentages of identity to Rep and Mob proteins of staphylococci and other bacteria deposited in the databases were detected.

C*onclusions*: Both plasmids, pKKS966 and pKKS49, appeared not to be typical staphylococcal plasmids. The homology to larger plasmids that harbour the genes *apmA* and/or *dfrK* was limited to these resistance genes and their immediate up- and downstream regions and thus suggested that these small plasmids were not integrated into larger plasmids.

Introduction

During recent years, several novel resistance genes, including the trimethoprim resistance gene *dfrK* and the apramycin resistance gene *apmA*, have been identified among methicillin-resistant *Staphylococcus aureus* (MRSA) of clonal complex (CC) 398 from animals.[1-3] As most of these novel resistance genes have been identified in MRSA isolates from pigs,[3] it appears as if there could be a potential porcine reservoir of antimicrobial resistance genes. Both genes, *dfrK* and *apmA*, were usually co-located with other resistance genes on multiresistance plasmids of ca. 40 kb.[1-3] The *dfrK* gene was commonly linked to the tetracycline resistance gene *tet*(L) as confirmed for plasmids in MRSA of sequence type (ST) 125 of human origin[4] or MRSA CC398 from pigs,[5] dairy cattle,[6] or food of poultry origin.[7] Some of these *tet*(L)-*dfrK*-carrying plasmids also carried additional resistance genes such as *cfr* and *aadD* (also known as *ant(4')-Ia*)[4] or *aadD* and *vga*(C).[3] In addition, *dfrK* [without *tet*(L)] has been identified as part of transposon Tn*559*, which was first detected in a porcine methicillin-susceptible *S. aureus* CC398.[8] The origin of both genes, *dfrK* and *apmA*, is unknown and analysis of their genetic environment did not reveal their source and how they became incorporated in the aforementioned multiresistance plasmids.

The aims of this study were to identify small staphylococcal plasmids that carry the trimethoprim resistance gene *dfrK* or the apramycin resistance gene *apmA* as sole resistance gene. Analysis of such plasmids for their structure and organisation might provide insight in whether these small plasmids could have acted as precursors of large multiresistance plasmids that carry *apmA* and/or *dfrK* in addition to other resistance genes.

Material and methods

To find out whether there are plasmids that carry only *dfrK* or *apmA*, trimethoprim- or apramycin-resistant staphylococcal isolates from the strain collections of both participating institutions were screened for the presence of the genes *dfrK* and *apmA* by previously described PCR assays.[2,5] Plasmids were prepared and transferred into *S. aureus* RN4220 via electrotransformation. Transformants were selected on trimethoprim- (30 mg/L) or apramycin-containing (20 mg/L) media. The plasmids were digested with suitable enzymes, cloned into pBluescript II SK+ and sequenced completely. Two small plasmids, one harbouring *dfrK* and the other harbouring *apmA*, were identified. Their sequences have been deposited in the EMBL database under accession numbers FN677368 (pKKS966) and HE611647 (pKKS49).

Results and discussion

The *dfrK*-carrying trimethoprim resistance plasmid pKKS966

The *dfrK*-carrying plasmid pKKS966 was isolated from a *Staphylococcus hyicus* subsp. *hyicus* isolate obtained from a sow with a skin infection during the BfT-GermVet study conducted between 2004 and 2006 in Germany. The isolate showed resistance to trimethoprim (MIC ≥ 512 mg/L) and enrofloxacin (MIC 4 mg/L). Plasmid pKKS966 had a size of 4,957 bp and a GC content of 36.1%. Sequence analysis identified five open reading frames (Figure 1). The first reading frame coded for a potential plasmid replication protein of 219 amino acids (aa). The Rep protein was next related to Rep proteins of small plasmids isolated from *Selenomonas ruminantium* (201 aa, 48% identity, accession no. NP_862700) or from *Bacillus mycoides* (186 aa, 41%, NP_981975.1). The second reading frame was the *dfrK* gene which encoded a 163 aa

dihydrofolate reductase. This *dfrK* gene was identical to previously identified, plasmid-located *dfrK* genes from MRSA ST398 and ST125.[1-3] It differed in six aa from a DfrK of an equine *S. aureus* isolate (accession no. CBL80435.1) and in seven aa from the DfrK located on Tn*559* from a methicillin-susceptible *S. aureus* ST398 isolate.[8] The *dfrK* flanking regions in pKKS966 showed homology to plasmid pKKS2187[1] for 166 bp in the *dfrK* upstream and for 377 bp in the *dfrK* downstream part. To the best of our knowledge, this is the first description of the *dfrK* gene in a staphylococcal species other than *S. aureus*. The three remaining reading frames of pKKS966 coded for Mob proteins. The *mobA* gene, encoded a putative relaxase of 382 aa with the best matches to a protein from a whole genome sequence of *Staphylococcus hominis* (321 aa, 56%; accession no. ZP_04060076.1), but also to proteins encoded by plasmids from *Staphylococcus epidermidis* (337 aa, 51%, AAD02405.1) or *S. aureus* (336 aa, 51%, EHT35286.1). The *mobC* gene encoded a protein of 105 aa which is most closely related to a protein from *Lactobacillus malefermentans* (123 aa, 52%, ZP_09441588.1). In contrast, the MobB protein of 182 aa exhibited only 29% aa identity to a MobB protein of *Pediococcus pentosaceus* (189 aa, AAD25895).

The *apmA*-carrying apramycin resistance plasmid pKKS49

The *apmA*-carrying plasmid pKKS49 originated from a MRSA ST398 isolate obtained from a dust sample taken in a holding with breeding pigs in Portugal as part of the EU baseline study. The isolate was resistant to apramycin (MIC ≥ 32 mg/L), tetracycline (MIC 64 mg/L), clindamycin (MIC ≥ 128 mg/L), tiamulin (MIC ≥ 128) and oxacillin (16 mg/L). In addition to pKKS49, it also harboured the small *vga*(C)-carrying plasmid pCPS49.[9] Plasmid pKKS49 had a size of 4,809 bp and a GC content of 38.6%. Five reading frames were identified (Figure 1). The first reading frame coded for a potential

plasmid replication protein of 216 aa. This Rep protein was distantly related to so far known Rep proteins with the best match of 71% aa identity to the Rep protein of plasmid pKKS966. Three in part overlapping reading frames for a 382 aa MobA protein, a 182 aa MobB protein and a 109 aa MobC protein were detected. The MobA, MobB and MobC proteins showed best matches of 82%, 75% and 77% aa identity to the respective proteins encoded by the *mob* genes of plasmid pKKS966. The sequence identities to pKKS966 of these two gene regions are shown in Figure 1. The fifth reading frame encoded a 274 aa protein that differed by 12 aa from the same-sized ApmA protein of plasmid pAFS11 from bovine MRSA ST398.[2] Analysis of the sequences flanking this *apmA* gene variant identified homology to plasmid pAFS11 for only 72 bp in the *apmA* upstream and 64 bp in the *apmA* downstream region. While *dfrK* has been described recently in isolates from Spain,[3,10] this is the first identification of the novel gene *apmA* in an isolate not originating from Germany.

The results of this study confirmed that the novel resistance genes *dfrK* and *apmA* do not only occur on large multiresistance plasmids, but also on small resistance plasmids that do not carry additional resistance genes. These plasmids showed an overall organization, which is similar to that of small staphylococcal resistance plasmids.[7] However, a closer look at the similarities on aa level revealed that the pKKS966- and pKKS49-associated Rep and Mob proteins are only distantly related to corresponding proteins of staphylococci. In addition, the observation that the GC contents of 36.1% (pKKS966) or 38.6% (pKKS49) are higher than the GC content of sequenced staphylococcal genomes, *Staphylococcus epidermidis* RP62A (32.1%), *Staphylococcus haemolyticus* JCSC1435 (32.8%), *Staphylococcus aureus* N315 (32.8%), *Staphylococcus saprophyticus* ATCC15305 (33.2%) and *Staphylococcus carnosus* TM300 (34.6%) might support the assumption that these two plasmids do not originate

from staphylococci.[11] Comparative analysis of the *dfrK*-flanking regions in plasmids pKKS966 and pKKS2187 and the *apmA*-flanking regions in plasmids pKKS49 and pAFS11, respectively, did not identify specific sequences or structures which might point towards recombination processes between the small plasmids and the corresponding larger *dfrK*- or *apmA*-carrying plasmids. As such, it is questionable whether these two small plasmids acted as sources for the incorporation of *dfrK* or *apmA* into larger plasmids.

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Transparency declarations

None to declare.

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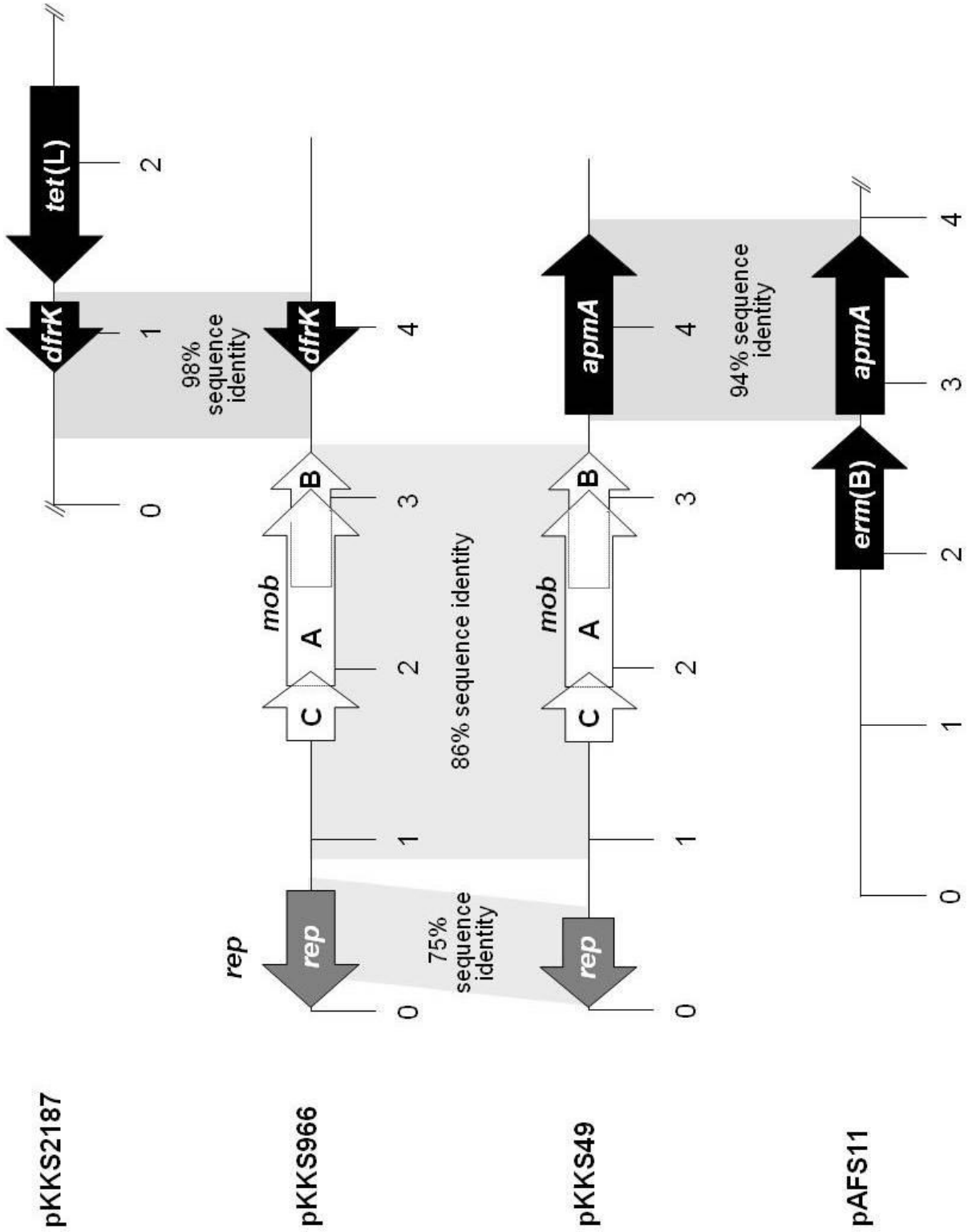
Staphylococcus aureus strains in a Spanish hospital. *J Antimicrob Chemother* 2012; **67:** 330-4.

11 Rosenstein R, Nerz C, Biswas L *et al*. Genome analysis of the meat starter culture bacterium *Staphylococcus carnosus* TM300. *Appl Environ Microbiol* 2009; **75:** 811-22.

Figure 1. Maps of the plasmids pKKS966 (FN677368) and pKKS49 (HE611647) in comparison to parts of the *dfrK*-carrying plasmid pKKS2187 (FN207105)[1] and the *apmA*-carrying plasmid pAFS11 (FN806789).[2] The arrows indicate the extents and directions of transcription of the genes *dfrK* (trimethoprim resistance), *tet*(L) (tetracycline resistance), *rep* (plasmid replication), *mobA, mobB, mobC* (plasmid recombination/mobilization), *apmA* (apramycin resistance) and *erm*(B) (macrolide/lincosamide/streptogramin B resistance). Resistance genes are coloured in black, *mob* genes in white and *rep* genes in grey. The region of homology between the plasmids is marked by grey shading. A distance scale in kb is given below each map. The maps of plasmids pKKS966 and pKKS2187 have been redrawn from the corresponding database entries to match the reading frames in pKKS49.


pKKS2187
tet(L)
dfrK
0
1
2
98% sequence identity
pKKS966
dfrK
rep
rep
mob
A
B
C
0
1
2
3
4
75% sequence identity
86% sequence identity
pKKS49
rep
mob
A
B
C
apmA
0
1
2
3
4
94% sequence identity
pAFS11
erm(B)
apmA
0
1
2
3
4

Chapter 11

Detection of the novel *vga*(E) gene in methicillin-resistant *Staphylococcus aureus* CC398 isolates from cattle and poultry

Hauschild T, Feßler AT, Kadlec K, Billerbeck C and Schwarz S

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doi:1093/jac/dkr446

Chapter 12

Target gene mutations among methicillin-resistant Staphylococcus aureus and methicillin-susceptible S. aureus with elevated minimum inhibitory concentrations of enrofloxacin obtained from diseased food-producing animals or food of animal origin

Hauschild T, Feßler AT, Billerbeck C, Wendlandt S,

Kaspar H, Mankertz J, Schwarz S and Kadlec K

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doi: 10.1093/jac/dks104

Chapter 13

High throughput Multiple Locus Variable Number of Tandem Repeat analysis (MLVA) of *Staphylococcus aureus* from human, animal and food sources

Sobral D, Schwarz S, Bergonier D, Brisabois A, Feßler AT, Gilbert FB, Kadlec K, Lebeau B, Loisy-Hamon F, Treilles M, Pourcel C and Vergnaud G

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doi:10.1371/journal.pone.0033967

Chapter 14

Discussion

14.1 General considerations

Among dairy cattle, mastitis is one of the economically most important diseases (Kossaibati and Esslemont, 1997). Mastitis is usually treated with antimicrobial agents and, besides other factors, the therapeutic success can be compromised by antimicrobial resistance (Barkema et al., 2006). *S. aureus* and CoNS play an important role as bovine mastitis pathogens (Barkema et al., 2006; Pyörälä and Taponen, 2009; Sampimon et al., 2009) and resistance against a broad range of antimicrobial agents has been described to occur in staphylococci (Lyon and Skurray, 1987; Werckenthin et al., 2001; Jensen and Lyon, 2009; Monecke et al., 2011). During recent years methicillin resistance among staphylococci of animal origin has gained particular attention (Voss et al., 2005; Witte et al., 2007; Leonard and Markey, 2008; Nemati et al., 2008; Kadlec et al., 2009; Cuny et al., 2010; Loeffler and Lloyd, 2010; Vanderhaeghen et al., 2010), but so far there are only limited studies on MRSA and MRCoNS from cattle available.

The identification and comprehensive characterization of methicillin-resistant staphylococci is a key component which enables the identification of the genomic relationships of the isolates present. As such, it allows the differentiation of clones from sporadic isolates present in an animal species in a certain geographical region, but also present in different hosts and/or different geographical regions. The use of appropriate typing methods is an indispensable requirement to gain insight into the epidemiological situation and to determine possible reservoirs and transmission routes (Schwarz et al., 2003).

14.2 Comparative characterization of LA-MRSA isolates from cases of bovine mastitis as well as non-bovine LA-MRSA isolates for their genetic relationships, virulence and resistance properties

The first description of MRSA causing bovine mastitis dated back in the 1970s and the transfer of human strains to cattle was most likely (Devriese et al., 1972; Devriese and Hommez, 1975). During the next three decades, people did not pay much attention to the few oxacillin-resistant MRSA isolates occasionally identified from cases of bovine mastitis (Craven et al., 1983; Watts and Salmon, 1997; Lange et al.,1999; De Oliveira et al., 2000). With the detection of MRSA CC398 in pigs and other animal species since 2004 (Voss et al., 2005; Nemati et al., 2008; Kadlec et al., 2009; Argudín et al., 2010; Graveland et al., 2010; Spohr et al., 2010; Vanderhaeghen et al., 2010; Argudín et al., 2011; Graveland et al., 2011), MRSA from cases of mastitis in dairy cattle gained renewed attention although only a few cases had been reported (Juhász-Kaszanyitzky et al., 2007; Monecke et al., 2007; Türkyilmaz et al., 2010). In the first study on mastitis-associated MRSA from Germany, 25 bovine MRSA isolates from as well as two human isolates from dairy farm workers of one of the 17 dairy farms involved in the study were investigated for their genetic relationship, their resistance and virulence properties (Chapter 2).

All isolates belonged to the clonal complex 398, which has been described as livestock-associated (Lindsay, 2010; Köck et al., 2010; Monecke et al., 2011). This is in contrast to previous reports on MRSA from bovine mastitis cases, where the isolates resembled mainly those of human origin (Devriese and Hommez, 1975; Juhász-Kaszanyitzky et al., 2007; Monecke et al., 2007; Türkyilmaz et al., 2010). Only one of the two MRSA isolates in the study of Monecke et al. (2007) belonged to the clonal complex 398. More recent studies reported the presence of MRSA CC398 among cattle in Belgium and The Netherlands (Graveland et al., 2009; Vanderhaeghen et al., 2010). The characterization of the MRSA CC398 isolates in our study showed a high variability in their ApaI macrorestriction patterns (n=9) and resistance pheno- (n=10) and genotypes (n=11) (Chapter 2), these results are similar to those of MRSA isolates from diseased pigs (Kadlec et al., 2009) and are also in accordance with the results of Argudín et al. (2011), where 33 resistance profiles and

35 Crf9I macrorestriction patterns among 100 ST398 isolates (from healthy and diseased pigs, dust samples from pig farms, milk and meat) were detected. The 25 bovine and the two human MRSA CC398 isolates carried the genes for α and δ haemolysins, but were negative for all other major virulence genes and all enterotoxin genes tested (Chapter 2). Again, this observation corresponded closely to the data published by Argudín et al. (2011), but differed slightly from those of Kadlec et al. (2009), who identified the genes for enterotoxin B and enterotoxins K + Q in one and in three of 54 isolates from diseased pigs, respectively.

In a study on 32 MRSA isolates from food of poultry origin, 28 of the isolates belonged to CC398, which also showed a high variability in their resistance pheno- and genotypes (Chapter 3). Like the isolates from bovine mastitis they were also negative for all the enterotoxin genes tested, whereas the remaining four isolates belonging to CC5 or CC9 were positive for the enterotoxin gene cluster (*egc*), which comprises the genes for the enterotoxins G, I, M, N, O and U. The genes *lukF*-PV and *lukS*-PV coding for the Panton-Valentine leucocidin have only been described rarely in MRSA CC398 (van Belkum et al., 2008; Stegger et al. 2010), but have not been detected among the 25 bovine MRSA, the two human MRSA (Chapter 2) and the 32 MRSA isolates from food of poultry origin (Chapter 3).

The results of the susceptibility testing revealed 10 different resistance patterns among the 25 bovine and two human CC398 isolates, with 11 isolates (40.7%) being only resistant against β-lactams and tetracyclines (Chapter 2). Among the isolates from poultry meat and poultry meat products, only one (3.6%) of the 28 CC398 isolates showed solely resistance against these two classes of antimicrobial agents (Chapter 3). In total, 20 different resistance patterns were observed among the MRSA isolates from food of poultry origin. In the study by Kadlec et al. (2009), 22 resistance patterns were observed with 14 of the 54 isolates (25.9%) being only resistant against β-lactams and tetracyclines. Analysis of the resistance genotypes of the bovine MRSA isolates showed the presence of numerous resistance genes in various combinations. In addition to the *mecA* gene, the resistance genes *blaZ*, *tet*(M), *tet*(K), *tet*(L), *erm*(A), *erm*(B) *erm*(C), *erm*(T), *aacA-aphD*, *aphA3*, *aadD*, *apmA*, *spc*, *fexA*, *dfrK*, *vga*(A), *vga*(C), *vga*(E) were detected among the MRSA

isolates from bovine mastitis and food of poultry origin (Chapters 2, 3, 9, and 11). The finding of a high variety of resistance genes in different combinations is also in accordance with the study on MRSA CC398 from pigs by Kadlec et al. (2009).

The observed differences in the percentages of isolates showing only resistance against β-lactams and tetracyclines might point towards different situations regarding the resistance properties of MRSA CC398 in different populations. The fact that the same methodology and mainly the same test panel of antimicrobial agents have been used makes it easier to compare the results of the study by Kadlec et al. (2009) and the studies described in Chapter 2 and Chapter 3. However, there are still some limitations for the comparison that should be noted. The investigation of 25 bovine MRSA isolates from 17 dairy farms does not allow to draw conclusions on the prevalence of MRSA associated with bovine mastitis in Germany as these isolates were not obtained during a representative national monitoring program, but from samples sent to specific diagnostic laboratories. The analysis of 720 bovine *S. aureus* isolates obtained during the National Resistance Monitoring Program GE*RM*-Vet Program in 2008-2009 revealed only eleven (1.5%) MRSA isolates, all of which were from cases of mastitis (Billerbeck et al., 2011). These data showed that the MRSA prevalence in cattle was comparatively low. Ten of the MRSA isolates investigated by Billerbeck et al. (2011) belonged to CC398, whereas the remaining isolate showed MLST type ST9. Four (40%) of the MRSA CC398 isolates were only resistant against β-lactams and tetracyclines and all MRSA CC398 isolates were negative for the enterotoxin genes included in the StaphyType DNA microarray (Billerbeck et al., 2011). As such, these results are in accordance with the results of the bovine MRSA isolates investigated in Chapter 2.

The MRSA isolates from food of poultry origin were collected during a small-scale study in Rhineland-Palatinate and showed a high MRSA prevalence with > 37 % of the samples being MRSA-positive (Chapter 3). As stated in the corresponding publication, this study presented only a time-limited snapshot of the presence of MRSA in fresh chicken and turkey meat as well as in the corresponding products sold in a specific part of Germany, and it is uncertain in how far the results found in

the federal state Rhineland-Palatinate can be extrapolated to other federal states in Germany or to Germany in general.

Since some studies suggest the possibility of transmission of *S.aureus*/MRSA via food and the gastrointestinal tract (Moritz and Smith, 2011; Valentin-Domelier et al., 2011), the detection of the transmission or contamination routes of MRSA, particularly with regard to food producing animals and food of animal origin, is of major relevance. In this context, MRSA associated with bovine mastitis might also play an important role for the possible contamination of raw milk and milk products. Moreover, poultry meat or poultry meat products contaminated with MRSA may also allow a transfer of the isolates to the consumer. In this regard, undercooked meat, but also – more importantly – cross-contamination in the kitchen play important roles.

14.3 Characterization of LA-MRSA isolates from humans and animals in Dutch dairy farms

The analysis of the 25 bovine and the two human MRSA isolates showed that both human isolates, obtained from farm personal, were indistinguishable from the three bovine isolates of the same farm. This observation points towards a transmission of MRSA CC398 isolates between humans and cattle (Chapter 2). A transmission of MRSA ST1 (eight isolates with indistinguishable PFGE patterns) between cattle and humans has been described previously (Juhász-Kaszanyitzky et al., 2007). Moreover, the presence of MRSA CC398 has also been described in different animal species as pigs, cattle, poultry, horses, dogs, rats and humans and therefore suggests a transmission between different hosts (Voss et al., 2005; Nemati et al., 2008; Kadlec et al., 2009; Nienhoff et al., 2009; van de Giessen et al., 2009; Graveland et al., 2010; Spohr et al., 2010; Vanderhaeghen et al., 2010; Argudín et al., 2011; Graveland et al., 2011). Spohr et al. (2010) investigated three dairy farms in Baden-Württemberg, with one of them also keeping pigs. They found the isolates from cows, humans and – if available – pigs being indistinguishable or closely related. Since there is not much information available about the transmission of MRSA CC398 between cattle, other animal species and humans living on the same

farm, 26 Dutch dairy farms were investigated by analyzing the MRSA isolates obtained from cattle, humans and other animal species present on the respective farms (Chapter 4). The characterization of the 125 MRSA isolates included in this study revealed different situations for the single farms. While MRSA isolates, which were indistinguishable by their *spa* type, SCC*mec* type, macrorestriction pattern, and resistance pattern, were seen among humans and different animal species within some farms, there were other farms where several MRSA subtypes, which differed in one or more of the aforementioned characteristics, were present in humans and animals. Holmes et al. (2010) noted that different *spa* types can be present among isolates that were indistinguishable by their PFGE patterns and vice versa. The presence of different *spa* types among isolates with the same macrorestriction pattern has also been observed for the isolates of some farms in our study (e.g. farms F and S). Therefore a reasonable combination of different methods should be used to increase the discriminatory power and to compensate the limitations of single typing methods. For this reason a combination of ApaI-PFGE, *spa* and SCC*mec* typing, supplemented with the respective resistance patterns was used for the characterization of the MRSA isolates in Chapter 4.

The observation that different MRSA subtypes were present within single farms is in accordance with a study by Bosch et al. (2010) who found various MRSA CC398 subtypes, which differed in their *spa* types and/or Cfr9I macrorestriction patterns, in single pig farms. Spohr et al. (2010) identified indistinguishable or closely related MRSA isolates, based on the analysis of their *spa* types and resistance patterns, within three dairy farms in Southwest Germany. The observed heterogeneity of the MRSA isolates could possibly be explained by several importations of different MRSA strains within the farms or the rapid evolution of the original MRSA strains in different subtypes. Moreover horizontal resistance gene transfer, which might change the resistance patterns, could occur between MRSA and MSSA/CoNS, which also play a role as bovine mastitis pathogens (Werckenthin et al., 2001; Pyörälä and Taponen, 2009; Sampimon et al., 2009).

The finding of the same MRSA subtypes among different animal species and humans suggest the likelihood of MRSA transmission between different hosts within

farms. Even if cattle and pigs are not necessarily in direct contact, farm workers who take care of different animals, or dogs, which have contact to the other animals on the farms, may act as transfer vehicles. Another source for transmission might be rats living on the farms, especially since the presence of MRSA CC398 has already been described among rats on pig farms (van de Giessen et al., 2009).

A recent study investigated risk factors for transmission of MRSA CC398 between humans and cattle (Graveland et al., 2010). They found that human MRSA carriage was strongly associated with the intensity of animal contact and with the number of MRSA positive animals on the farm. Moreover, better farm hygiene was associated with a lower prevalence of MRSA (Graveland et al., 2010). Longitudinal studies – similar to the one on methicillin-resistant *S. pseudintermedius* in households (Laarhoven et al., 2011) – including an appropriate characterization of the isolates could possibly identify the reasons for the diversity among MRSA CC398 isolates present within and between farms. Finally, the transmission routes of MRSA CC398 into and between dairy farms as performed for different kinds of pig farms by van Duijkeren et al. (2008) could also elucidate the presence of different MRSA subtypes on the same farm.

14.4 Characterization of MRCoNS isolates from cases of bovine mastitis in Germany for their genetic relationships and resistance properties with particular reference to the correct assessment of methicillin resistance

Besides *S. aureus*, CoNS play an important role as mastitis pathogens (Pyörälä and Taponen, 2009; Sampimon et al., 2009), and penicillin G is commonly used for the treatment of mastitis cases caused by penicillin-susceptible pathogens (Pyörälä and Taponen, 2009). In comparison to *S. aureus*, there is even less information on methicillin resistance among CoNS from bovine mastitis available. During a study by Lüthje and Schwarz (2006), two oxacillin-resistant and *mecA*-positive isolates with MICs of 8 and ≥ 128 mg/L, were detected among 298 CoNS isolates. This observation indicates a low prevalence of methicillin resistance among CoNS isolates from bovine subclinical mastitis. In addition, it should be noted that the interpretive

criteria for oxacillin given in the actual CLSI document M31-A3 are not veterinary-specific breakpoints but adopted from human medicine (CLSI, 2008). Moreover, the CLSI approved clinical breakpoints for oxacillin and the interpretive criteria for the cefoxitin disk test proved to be not appropriate for the detection of methicillin resistance among *S. peudintermedius* (Bemis et al., 2009; Weese et al., 2009).

These data indicated that clinical breakpoints for the correct assessment of methicillin resistance among CoNS should be carefully considered. This was done for 121 CoNS isolates from bovine mastitis (Chapter 5). The findings of this study showed that oxacillin disk diffusion and cefoxitin disk test were more appropriate to correctly predict the presence of the *mecA* gene, which was used as gold standard, than oxacillin broth microdilution. Ten of the 13 isolates with oxacillin MICs of 0.5 and 1 mg/L, classified as resistant by the CLSI breakpoint (CLSI, 2008), were *mecA*-negative. These data were in accordance with the situation among *S. pseudintermedius* (Bemis et al., 2009, Weese et al., 2009) and the observation in human medicine that *mecA*-negative CoNS, others than *S. epidermidis*, may have oxacillin MICs of 0.5 – 2 mg/L (CLSI, 2011). Based on the results obtained from the study in Chapter 5, the forthcoming CLSI document M31-A4, which is intended to be published in 2012, will include the recommendation to test CoNS with oxacillin MICs of 0.5 and 1 mg/L for the presence of the *mecA* gene or PBP2a before reporting them as methicillin-resistant.

Moreover the further characterization of the 15 *mecA*-positive MRCoNS revealed that all five *S. haemolyticus* isolates carried SCC*mec* elements of type V and exhibited higher oxacillin MICs of ≥16 mg/L, which was also seen for the single *S. saprophyticus* isolate with SCC*mec* type III. In contrast, the eight *S. epidermidis* isolates and the single *S. capitis* exhibited lower oxacillin MICs of 1 – 4 mg/L. All these isolates, except one *S. epidermidis* with a non-typeable SCC*mec* element, carried a SCC*mec* type IV element. These findings might suggest a correlation of the SCC*mec* element and/or the bacterial species with the conferred level of resistance.

The finding that seven *S. epidermidis* and all five *S. haemolyticus* isolates carried SCC*mec* elements of type IV and V, respectively, is in accordance with observations from human isolates (Ruppé et al., 2009). A recent study on methicillin-

resistant *S. haemolyticus* from immunocompromised hospital patients revealed different SCC*mec* elements among the *S. haemolyticus* isolates with SCC*mec* type V being most prevalent (Bouchami et al., 2011). In contrast to our findings, the results of Bouchami et al. (2011) did not reveal a correlation between the oxacillin MICs and the respective SCC*mec* elements. The finding of isolates harboring more than one SCC*mec* element as well as the detection of various unnamed and non-typeable SCC*mec* elements (Garza-González et al., 2010a; Garza-González et al., 2010b; Zong et. al., 2011) is in accordance with the presence of an additional *ccrA4/B4* among two *S. epidermidis* isolates with SCC*mec* type IV and the presence of a non-typeable SCC*mec* element. In comparison to our study, Zhang et al. (2009) found methicillin resistance among *S. sciuri*, *S. lentus*, *S. xylosus* and *S. cohnii* isolates from cattle obtained in the U.S.A..

Moreover, it should be noted that MRCoNS might play a role as a reservoir for SCC*mec* elements which can be exchanged with CoNS and *S. aureus*. This is supported by the finding of the same SCC*mec* elements in methicillin-resistant isolates of different CoNS species within single farms and the identification of SCC*mec* IVa elements in *S. epidermidis* that were highly homologous to those of *S. aureus* (Barbier et al., 2010). While only one isolate in our study, the single *S. saprophyticus*, carried a SCC*mec* element of type III, Zhang et al. (2009) found SCC*mec* type III in 19 of 60 MRCoNS of different CoNS species. Since SCC*mec* elements of type III are also common in HA-MRSA, these findings might suggest a horizontal gene transfer between MRSA of human origin and MRCoNS of animal origin (Zhang et al., 2009).

14.5 Proposal of interpretive criteria for cefoperazone susceptibility testing of bovine mastitis pathogens (including LA-MRSA and MRCoNS)

The correct assessment of mastitis pathogens for their susceptibility/resistance is currently hampered by the lack of harmonized test conditions and veterinary-specific interpretive criteria. For the susceptibility testing of bovine mastitis pathogens, there are so far only CLSI-approved, veterinary-specific clinical

breakpoints for ceftiofur, pirlimycin and the combination penicillin/novobiocin available (CLSI, 2008). Cefoperazone is a third generation cephalosporin approved for the treatment of bovine mastitis. It is available either as 250 mg or 100 mg compound for intramammary application.

Although cefoperazone is widely used, problems with regard to the interpretation of cefoperazone *in-vitro* susceptibility testing data have been encountered in routine mastitis diagnostic laboratories. This is mainly due to the facts that (i) different disks charged with either 10 µg, 30 µg or 75 µg are commercially available for agar disk diffusion tests, and (ii) no veterinary-specific interpretive criteria for either disk diffusion or MIC testing are currently available. The human clinical breakpoints from the CLSI document M100-S21 (CLSI, 2011) [MIC: ≤ 16 mg/L (susceptible), 32 mg/L (intermediate), ≥ 64 mg/L (resistant); zone diameters: ≥ 21 mm (susceptible), 16-20 mm (intermediate), ≤ 15mm (resistant)] appear to be inadequate as they do not appropriately reflect the cefoperazone concentrations achievable by recommended dosing in the bovine udder. Concentrations of > 16 mg/kg milk can only be achieved for a very short period, but not for the required period of the dosing interval. In contrast, concentrations of > 2 mg/kg milk can be achieved for either the entire dosing interval of the higher strength (250 mg) cefoperazone compound or approximately 80% of the dosing interval of the lower strength (100 mg) compound (Hamann et al. 1986; Wilson and Gilbert 1986).

To make an educated guess for bovine mastitis-specific interpretive criteria of cefoperazone, a total of 1,086 target pathogens, which also comprised 188 *S. aureus* (including 17 MRSA) and 196 CoNS (including 15 MRCoNS), were tested in parallel for their cefoperazone MICs and the zone diameters using a 75 µg disk. Subsequently, MICs were plotted against zone diameters.

When the human clinical breakpoints (CLSI, 2011) were applied, none of the MRSA isolates was classified as resistant or intermediate by both methods, but three isolates were classified as susceptible. A similar situation was seen among the CoNS with one (0.5 %) and three (1.5 %) MRCoNS being classified as resistant and intermediate, respectively, one (0.5 %) MRCoNS isolate was present in the minor error category and the remaining ten MRCoNS were classified as susceptible.

Based on the pharmacological data, the clinical efficacy (Holmgren et al., 1985; Wilson et al., 1986; Wilson and Gilbert, 1986) and the microbiological data, a proposal was made for veterinary-specific breakpoints which classify members of the aforementioned species/groups as (a) susceptible to cefoperazone when their MIC is ≤ 2 mg/L and their zone diameters are ≥ 27 mm (staphylococci or *E. coli*) or ≥ 21 mm (streptococci), (b) intermediate when their MIC is 4 µg/ml and their zone diameters are 22 to 26 mm (staphylococci or *E. coli*) or 16 to 20 mm (streptococci), and (c) resistant when their MIC is ≥ 8 µg/ml and their zone diameters are ≤ 21 mm (staphylococci or *E. coli*) or ≤ 15 mm (streptococci) (Chapter 6).

Using these new breakpoints, 16 of the 17 MRSA isolates were classified as resistant with the remaining one being in the minor error category (resistant by MIC, intermediate by zone diameter). Thus, a comment for the diagnostic laboratory to consider MRSA isolates as resistant to cefoperazone appears to be justified. This is also in agreement with the recommendation given in Table 2C of the CLSI document M100-S21 to classify MRSA and MRCoNS as cefoperazone-resistant (CLSI, 2011). The five MRCoNS with MICs of ≥ 32 µg/ml were classified as resistant and the single MRCoNS isolate with a MIC of 16 µg/ml as minor error whereas the MRCoNS with cefoperazone MICs of 4 µg/ml and 2 µg/ml were classified as intermediate or susceptible/minor error, respectively. Our data suggest that cefoperazone MICs differ among the MRCoNS with regard to the SCC*mec* cassette and/or the staphylococcal species present. In Table 2C of the CLSI document M100-S21 (CLSI, 2011), a warning comment is given that “certain β-lactam antibiotics, including cephalosporins, may appear active *in-vitro* against methicillin (oxacillin)-resistant *S. aureus* and CoNS, but are not effective clinically”. Unless specific clinical efficacy studies have confirmed the clinical efficacy of cefoperazone against MRCoNS with the aforementioned low MICs, we suggest to classify all MRCoNS as cefoperazone-resistant.

14.6 Analysis of plasmid-borne resistances to protein biosynthesis inhibitors among staphylococci with particular reference to the identification of the molecular basis of apramycin resistance and combined resistance to lincosamides, pleuromutilins and streptogramin A antibiotics

Antimicrobial resistance in bacterial pathogens has a strong influence on the therapeutic options in veterinary medicine (Schwarz and Chaslus-Dancla, 2001; Schwarz and Werckenthin, 2001). Besides β-lactam antibiotics, which target the bacterial cell wall, protein biosynthesis inhibitors (PBIs) play an important role for the treatment of staphylococcal infections (Chapter 7). PBIs constitute a highly diverse group of antimicrobial agents which target either the 30S subunit of the ribosome, the 50S subunit of the ribosome, translational factors or the enzyme isoleucyl-tRNA synthetase (Lyon and Skurray, 1987; Schwarz et al., 2006; Jensen and Lyon, 2009).

The horizontal gene transfer, e.g. via plasmids and transposons, enables the spread of antimicrobial resistance genes within a species and also between different bacterial species (Lyon and Skurray, 1987; Schwarz and Chaslus-Dancla, 2001; Werckenthin et al., 2001). Various studies confirmed that antimicrobial resistance genes in staphylococci are often located on mobile genetic elements among which plasmids play the most important role. The overview of PBI resistance genes provided in Chapter 7 and their association with mobile genetic elements underline the gene acquisition capacities of MRSA, but also their role as donors in the spread of PBI resistance genes.

Besides PBI resistance genes that are common among staphylococci, several novel and unusual resistance genes have been described to occur on plasmids or transposons among MRSA CC398 (Chapter 8), including *cfr* (Kehrenberg et al., 2009), *erm*(T) (Kadlec and Schwarz, 2010a), *apmA* (Chapters 9 and 10), *vga*(C) (Kadlec and Schwarz, 2009b) and *vga*(E) (Schwendener and Perreten, 2011) (Chapter 11).

14.6.1 *apmA*-mediated apramycin resistance

The aminocyclitol antibiotic apramycin is approved for the treatment of colibacillosis in pigs, cattle, sheep, poultry and rabbits. In *Enterobacteriaceae*

apramycin resistance is mediated via the gene *aac(3)-IV*, which is commonly located on plasmids (Davies and O'Conner, 1978; Chaslus-Dancla et al., 1986; Threlfall et al., 1986; Wray et al., 1986; Chaslus-Dancla et al., 1991). Since apramycin is not licenced for the treatment of staphylococcal infections there is not much information on apramycin resistance in staphylococci available. Four, two and one isolates from diseased pigs (Kadlec et al. 2009), cases of bovine mastitis (Chapter 2) and a poultry meat product (Chapter 3), respectively, showed apramycin MICs of ≥ 32 mg/L. Apramycin resistance was associated with plasmids of ca. 40-kb, that showed undistinguishable or closely related restriction patterns. One such plasmid, obtained from the bovine MRSA isolate 11, was further analyzed to identify the gene responsible for apramycin resistance. This plasmid, designated pAFS11, also carried the resistance genes *aadD*, *tet*(L), *erm*(B) and *dfrK* and conferred – besides apramycin resistance – resistance against kanamycin, neomycin, tetracyclines, macrolides, lincosamides and trimethoprim when transferred into *S. aureus* RN4220. Cloning and deletion analysis identified the novel gene *apmA* in close proximity to the gene *erm*(B) on plasmid pAFS11 (Chapter 9). The co-location of *apmA* with other resistance genes on the same multiresistance plasmid can contribute to the persistence and spread of the *apmA* gene under selective pressure by any of the aforementioned antimicrobial agents.

Moreover, an *apmA* gene variant, whose product differed by 12 amino acids from the pAFS11-associated ApmA protein, was found on a small 4809-bp plasmid, designated pKKS49. Plasmid pKKS49 originated from a porcine MRSA ST398 from Portugal which had been detected during the European baseline study (Chapter 10). Interestingly, the homology of the two plasmids was limited to the *apmA* gene and 72 bp in its upstream region and 64 bp in its downstream region. Despite the limited homology, it should be noted that the detection of the *apmA* gene in two different countries might indicate a further distribution of this gene which might be detected, when screening for elevated apramycin MICs in staphylococci.

In contrast to the gene *aac(3)-IV* in Enterobacteriaceae, which confers cross-resistance between apramycin and the aminoglycoside gentamicin (Chaslus-Dancla et al., 1986; Threlfall et al., 1986; Wray et al., 1986; Chaslus-Dancla et al., 1991), the

gene *apmA* confers only an increased insensitivity to gentamicin. Analysis of the gentamicin MICs of *S. aureus* RN4220 transformants carrying *apmA* showed an increase in the gentamicin MICs to a maximum value of 8 mg/L – however, a gentamicin MIC of 8 mg/L classified the corresponding transformants as intermediate to gentamicin (CLSI, 2008; Chapter 9).

14.6.2 *vga*(E)-mediated resistance to pleuromutilins/lincosamides/streptogramin A

Combined resistance to pleuromutilins, lincosamides and streptogramin A was found to be commonly mediated via ABC-transporters encoded by *vga* gene variants. A major problem in phenotypic identification of the corresponding genes is that (i) no breakpoints for pleuromutilins and streptogramin A antibiotics applicable to staphylococci are available, (ii) streptogramin A (except for the extremely expensive virginiamycin M1) and streptogramin B antibiotics are not available separately for susceptibility testing – instead the combination quinupristin/dalfopristin, which is approved for human use, is commonly tested, and (iii) lincosamide resistance is not a true indicator as there are numerous other genes in staphylococci which confer lincosamide resistance.

Based on previous experimental experience (Kadlec et al., 2009, Chapter 2, Chapter 3), MICs of the pleuromutilin tiamulin of ≥ 16 mg/L were used as indicator to test the corresponding isolates for *vga* genes. Among the MRSA isolates tested, tiamulin resistance was detected in 8 (32%) of the 25 bovine (Chapter 2) and in 16 (50%) of the 32 MRSA isolates from food of poultry origin (Chapter 3). The genes *vga*(A) and *vga*(C) could be detected in five and one of the bovine isolates and in five and five of the isolates from food of poultry origin, respectively. Attempts to identify the genetic basis of pleuromutilin resistance in the remaining isolates was not successful, but confirmed that these isolates did neither harbor *vga*(A), *vga*(B), *vga*(C), or *vga*(D) nor showed plasmid-located pleuromutilin resistance. In 2011, Schwendener and Perreten (2011) described a novel pleuromutilin, lincosamide, streptogramin A resistance gene, *vga*(E), located on a transposon, designated Tn*6133*, in porcine MRSA ST398. PCR screening revealed the presence of the *vga*(E) gene and its location on transposon Tn*6133* in the remaining two and six

isolates from cattle and food of poultry origin, respectively (Chapter 11). Moreover, this gene could also be detected in a single clinical MRSA CC398 isolate from a turkey (Billerbeck et al., 2011). All *vga* genes detected so far in staphylococci of human and animal origin, including *vga*(A) with the variants *vga*$(A)_{LC}$ and *vga*(A)*v*, *vga*(B), *vga*(C) (Allignet and Solh, 1997; Haroche et al. 2000; Gentry et al.,2008; Novotná and Janata, 2006; Kadlec and Schwarz, 2009b) and the recently described *vga*(E) (Schwendener and Perreten, 2011), are associated with mobile genetic elements. This facilitates their dissemination across species and genus boundaries. As a consequence, prudent use of antimicrobial agents is strongly recommended to reduce the spread of antimicrobial resistance and to retain the efficacy of the currently available antimicrobial agents (Schwarz and Chaslus-Dancla, 2001; Schwarz and Werckenthin, 2001).

14.7 Analysis of fluoroquinolone resistance, mediated via target gene mutations

Besides various resistance genes, which are often located on mobile genetic elements (Chapters 7 and 8), antimicrobial resistance can also be mediated by mutations in genes that code for the cellular target site of the respective antimicrobial agents. This is a common mechanism in fluoroquinolone resistance (Takahashi et al., 1998; Hooper, 2008; Couto et al., 2008). Enrofloxacin was the first fluoroquinolone approved for veterinary medicine and is commonly used, because it is active against a wide spectrum of bacterial pathogens (Martinez et al., 2006; Lin and Davies, 2007). Unfortunately there are only CLSI approved clinical breakpoints for staphylococci from cats and dogs available (CLSI, 2008). Based on the aforementioned breakpoint, that classifies isolates of ≤ 0.5 mg/L as susceptible, enrofloxacin MICs of ≥ 1 mg/L were considered as elevated. Such MICs have been detected in 36 MRSA and MSSA isolates of animal or food origin investigated in different previous studies (Chapter 12). While all bovine MRSA isolates described in Chapter 2 had enrofloxacin MICs of ≤ 0.5 mg/L, two (18.2 %) of the 11 bovine MRSA isolates collected in the GE*RM*-Vet program 2008-2009 (Billerbeck et al., 2011) showed

enrofloxacin MICs of 1 and 4 mg/L. Twenty-four of the 36 isolates were assigned to CC398, six to CC5 and five to CC9, while the remaining isolate belonged to the novel sequence type ST2346. All 36 isolates had mutations in the quinolone resistance determining regions (QRDRs) of the genes *gyrA*, *gyrB*, *grlA* and/or *grlB* (Chapter 12) encoding the subunits for the enzymes DNA gyrase and DNA topoisomerase IV (Takahashi et al., 1998; Hooper, 2008). Mutations in two or three of the genes *gyrA*, *gyrB*, *grlA* and *grlB* were detected in all but two isolates. The latter two had only a single mutation in the *grlB* gene (Chapter 12). All isolates with enrofloxacin MICs of ≥ 4 mg/L contained the GrlA substitution Ser80Phe and one of the GyrA substitutions, Ser84Leu or Ser84Ala. This is in accordance with the results of Takahashi et al. (1998), who found the two substitutions, Ser80Phe in GrlA and Ser84Leu in GyrA, being present in all isolates with high ofloxacin MICs. Isolates with enrofloxacin MICs of 1 or 2 mg/L showed the GrlA substitution Ser80Phe, Ser80Tyr or Ser80Leu (Chapter 12). Moreover, except for the two porcine isolates with the single mutation and another isolate with a Glu88Asp substitution in GyrA, the remaining 15 isolates with MICs of 1 or 2 mg/L had mutations in the genes *grlA* and *grlB*, but not in *gyrA* (Chapter 12). This is in accordance with the observation that mutations in both target genes *grlA* and *gyrA* result in higher MIC values than single mutations (Tanaka et al. 2000).

The Glu422Asp substitution in the GrlB was present in all MRSA CC398 isolates regardless of their origin, whereas this substitution was not seen in any of the CC5 or CC9 isolates tested. Two types of point mutations were detected in the *norA* promotor region, both of which did not affect the ribosome binding site, the -35 and -10 positions. It should be noted that no specific *norA* PCR-amplicon could be obtained from the ST2346 isolate. Moreover all MRSA CC398 isolates tested shared the same type of mutations in the *norA* gene (Chapter 12).

14.8 Investigation of a refined Multiple Locus Variable Number of Tandem Repeat analysis (MLVA) approach for its ability to discriminate between LA-MRSA strains

The Multiple Locus Variable Number of Tandem Repeat (VNTR) analysis (MLVA) analysis is a cost-efficient typing method that is appropriate for the typing of large numbers of isolates and has already been used successfully for the typing of *S. aureus* isolates in different approaches (Sabat et al., 2003; Rasschart et al., 2009; Schouls et al., 2009). Since VNTR regions are used instead of highly conserved regions of housekeeping genes as in MLST, MLVA typing might be more appropriate for short-term epidemiological investigations (Pourcel et al., 2009). Recently, the automated sequencing of fluorescence labeled PCR products was described as an alternative to the usage of agarose gel electrophoresis for the detection of MLVA results, enhancing the accuracy and portability of this method (Schouls et al., 2009).

A novel approach, here termed MLVA-16_{Orsay} analyzing 16 targets, was compared to the MLVA scheme described by Schouls et al. (2009), using eight targets (Chapter 13). The comparison of the results for a subset of 90 isolates from the HARMONY collection revealed forty-nine types using the 16 loci compared to forty-one types using the eight loci approach. Despite the missing direct comparison with PFGE, which has been investigated in previous studies (Sabat et al., 2003; Rasschart et al. 2009; Schouls et al., 2009; Holmes et al., 2010; Rivero-Perez et al. 2010), these results show a high discriminatory power for the typing of *S. aureus* isolates. The clustering of the MLVA types showed a good correlation to the MLST clusters, which is in accordance with previous reports (Pourcel et al., 2009; Rasschart et al., 2009; Schouls et al., 2009).

The ability of the MLVA typing to discriminate *S. aureus* isolates from different sources was analyzed using 106 human clinical isolates, 98 isolates from animals, 34 isolates from food and 13 isolates associated with food poisoning (Chapter 13). The finding that the isolates of animal origin revealed not only clonal complexes as CC9, CC97, CC133 and CC398, which are likely to be associated with animals (Guinane et al., 2010; Monecke et al. 2011) but also isolates belonging to well-known human CCs e.g. CC5, CC8, CC15 and CC45 (Monecke et al., 2011) indicates a possible

transmission of *S. aureus* between humans and animals as described previously (Juhász-Kaszanyitzky et al., 2007, Nienhoff et al., 2009). In contrast to the isolates from food poisoning, 32 of the 34 *S. aureus* isolates from food were associated with livestock (CC398, CC9 or CC133) (Monecke et al., 2011), and indicate animals as the most likely source of contamination (Chapter 13).

The analysis of the 56 CC398 isolates (Chapter 13) resulted in seven different *spa* types and 20 MLVA-16_{Orsay} types. For this collection, the *spa* typing revealed a comparatively low discriminatory power of D = 0.5929, while a distinctly higher discriminatory index D = 0.8682 was obtained with the MLVA-16_{Orsay} assay. Moreover, the MLVA-16_{Orsay} assay was able to further discriminate isolates that shared the same *spa* type. For example, seven MLVA-16_{Orsay} types were seen among the 24 isolates of *spa* type t011, whereas another nine MLVA-16_{Orsay} types were present among the 27 isolates of *spa* type t034. While the isolate with *spa* type t6574 exhibited the same MLVA-16_{Orsay} type as a single isolate with *spa* type t011, the remaining four isolates of *spa* types t899, t2346, t3041, or t5719 displayed individual MLVA-16_{Orsay} types. The results presented in (Chapter 13) confirmed that the MLVA-16_{Orsay} assay is a useful tool for genotyping of HA-MRSA, but also LA-MRSA isolates.

14.9 Conclusions

With regard to the aims of the present Ph.D. study as listed in Chapter 1, the following aims were accomplished:

(1) All MRSA isolates from cases of bovine mastitis as well as most of the MRSA isolates from poultry meat and poultry meat products in Germany represented CC398. These CC398 isolates showed a high variability in their genomic structure and their resistance pheno- and genotypes, but exhibited rather uniform virulence patterns in which major toxin genes were absent.

(2) The finding of indistinguishable MRSA CC398 isolates within different animal species and humans on single dairy farms from the Netherlands indicates that

a transfer of MRSA CC398 between humans and animals is likely to occur. In contrast, the observation that different MRSA CC398 subtypes were present among humans and different animal species might point towards different importation routes into the respective farms and/or a rapid diversification of the original strains.

(3) The analysis of MRCoNS isolates – as identified by phenotypic methods – for the correct assessment of methicillin resistance resulted in a CLSI-approved recommendation to investigate MRCoNS that exhibit oxacillin MICs of 0.5 and 1 mg/L for the presence of the *mecA* gene or the expression of the PBP2a protein before reporting them as methicillin-resistant.

(4) A proposal for interpretive criteria for cefoperazone susceptibility testing of bovine mastitis pathogens was made which took into account udder-specific pharmacological data, clinical efficacy in dairy cattle and susceptibility data of common mastitis pathogens. These interpretive criteria are intended for use in routine mastitis diagnostics and allow a much more reliable assessment of cefoperazone resistance as do breakpoints adopted from human medicine.

(5) The occurrence of transferable, mainly plasmid-borne genes that confer resistance to protein biosynthesis inhibitors among staphylococci was reviewed. Among them were a number of novel and uncommon resistance genes, some of which have been identified so far mainly (or exclusively) in MRSA. The first and so far only apramycin resistance gene in staphylococci, *apmA*, has been identified. This gene was located either on large multiresistance plasmids or on small plasmids that carried only the *apmA* gene. In addition, the recently described *vga*(E) gene, which has been found only in MRSA CC398 from swine, was detected among MRSA CC398 from cattle, poultry, and food of poultry origin.

(6) Target gene mutations mediating fluoroquinolone resistance were identified in MRSA and MSSA isolates from different sources including two bovine MRSA isolates. Regardless of their origin, CC398 isolates showed specific mutations that were not detected in CC9 or CC5 isolates.

(7) Finally, a refined Multiple Locus Variable Number of Tandem Repeat analysis (MLVA) approach was investigated for its ability to discriminate between LA-MRSA strains. This typing method proved to be a suitable tool for the further discrimination of LA-MRSA and proved to be able to occasionally differentiate CC398 isolates that shared the same *spa* type.

Chapter 15

Abstract

Abstract

Andrea Theresia Feßler (2012)

Comparative molecular analysis of methicillin-resistant isolates of *Staphylococcus aureus* and coagulase-negative *Staphylococcus* spp. from cases of mastitis among dairy cattle

During recent years methicillin-resistance among staphylococci from humans and animals have been the focus of many studies, but only limited information on methicillin-resistance among staphylococci from bovine mastitis was available at the beginning of this Ph.D. project. The aims of this study were to comprehensively characterize bovine methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant coagulase-negative staphylococci (MRCoNS) from cases of mastitis and to compare bovine MRSA CC398 isolates with those from other sources.

The genetic relationships and resistance properties of 25 bovine and two human MRSA isolates from 17 dairy farms in Germany were investigated. All isolates belonged to the clonal complex (CC) 398. Nine ApaI macrorestriction main patterns were exhibited. Three *spa* (t011, t034 and t2576), two SCC*mec* (IV, V, one isolate non-typeable) and five *dru* types (dt6j, dt10a, dt10q, dt11a, dt11ab) were observed. Susceptibility testing revealed ten resistance patterns. Besides the *mecA* gene which mediates methicillin resistance, the resistance genes *blaZ* (penicillins), *tet*(K), *tet*(L), *tet*(M) (tetracyclines), *erm*(A), *erm*(B), *erm*(C), *erm*(T) (macrolides, lincosamides, streptogramin B), *aacA-aphD*, *aphA3*, *aadD* (aminoglycosides), *spc* (spectinomycin), *fexA* (phenicols), *dfrK* (trimethoprim), *vga*(A) and *vga*(C) (pleuromutilins, lincosamides, streptogramin A) were detected. A similar situation was observed for 32 MRSA isolates from food of poultry origin, including 28 CC398 isolates with highly variable resistance pheno- and genotypes.

To investigate the situation on farm level, 125 MRSA CC398 isolates from different animal species and humans from 26 Dutch dairy farms were characterized. In total, eight *spa* types (t011, t034, t108, t567, t1184, t1451, t2287, t3934), two

SCC*mec* types (IV, V, and 10 non-typeable isolates), 12 resistance patterns and numerous ApaI macrorestriction patterns were observed. On the single farms different situations were seen. While indistinguishable MRSA CC398 isolates among humans and different animals were detected on some farms, different MRSA types or subtypes were present on other farms, suggesting different transmission/importation routes or the diversification of the MRSA isolates over time.

Since the breakpoints for the identification of methicillin resistance among coagulase-negative staphylococci (CoNS) were adopted from human medicine, 121 isolates from bovine mastitis were tested for the correct assessment of methicillin resistance using different methods according to CLSI recommendations. The *mecA* gene was present in 15 of the 16 isolates phenotypically classified as resistant. Oxacillin broth micodilution classified 10 *mecA*-negative isolates with oxacillin MICs of 0.5 and 1 mg/L as resistant, whereas all but one isolates were classified correctly by oxacillin and cefoxitin disk diffusion. Therefore, the *mecA* gene or its gene product should be detected before reporting CoNS with oxacillin MICs of 0.5 and 1 mg/L as methicillin-resistant. SCC*mec* typing of the 15 *mecA*-positive MRCoNS isolates revealed SCC*mec* elements of types III, IV and V, while one isolate was non-typeable.

These CoNS isolates were also used for a study developing a proposal for clinical breakpoints of cefoperazone, which comprised a total of 1,086 isolates from the U.S.A. and Germany, including *S. aureus*, CoNS, *Escherichia coli*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*. Based on the cefoperazone concentrations achievable in the bovine udder, the following breakpoints were suggested: susceptible [MICs of ≤ 2 mg/L; zone diameters of ≥ 27 mm (staphylococci/*E. coli*) and ≥ 21 mm (streptococci)], intermediate [MICs of 4 mg/L; zone diameters of 22-26 mm (staphylococci/*E. coli*) and 16-20 mm (streptococci)] and resistant [MICs of ≥8 mg/L; zone diameters of for ≤ 21 mm (staphylococci/*E. coli*) and ≤ 15 mm (streptococci)]. Regarding the detection of methicillin resistance, all MRSA isolates were correctly separated from the susceptible population, whereas cefoperazone MICs differed among the MRCoNS with regard to the SCC*mec* cassette and/or the *Staphylococcus* spp. present.

The current knowledge on (i) plasmid-mediated resistance against protein biosynthesis inhibitors among staphylococci as well as (ii) novel and uncommon antimicrobial resistance genes in livestock-associated LA-MRSA has been presented in two review articles. Moreover, resistance against apramycin and combined resistance against lincosamides, pleuromutlilins and streptogramin A have been studied in detail. So far, no information on apramycin resistance among staphylococci has been available. Studies of MRSA isolates from cattle (n=2), pigs (n=4) and food of poultry origin (n=1) with apramycin MICs of ≥ 32 mg/L identified a plasmid-borne gene, designated *apmA*, encoding a 274 amino acid protein with distinct homology to acetyltransferases. NsiI deletion of the *apmA* gene lead to a 16- to 32-fold decrease of the apramycin MICs, which confirmed that this gene mediated apramycin resistance. Moreover, an *apmA*-variant was detected on the small plasmid pKKS49 from a porcine MRSA ST398 isolate. Studies on the genetic basis of combined lincosamide, pleuromutilin and streptogramin A resistance identified – in addition to the genes *vga*(A) and *vga*(C) – the recently described *vga*(E) gene for the first time in MRSA CC398 from a turkey (n=1), cattle (n=2), and food of poultry origin (n=6).

The investigation of the genetic basis of elevated enrofloxacin MICs of ≥ 1 mg/L among 21 MRSA and 15 MSSA isolates of the clonal complexes CC398, CC9 and CC5 from pigs, cattle, poultry and food of poultry origin revealed different mutations in the respective target genes *grlA*, *gyrA*, *grlB* and/or *gyrB* as well as in the regulatory region of *norA*. Regardless of their origin, all CC398 isolates exhibited a Glu422Asp substitution in GrlB and a specific set of *norA* regulator mutations that were absent in the CC5 and CC9 isolates.

Moreover, a novel approach for the Multiple Locus Variable Number of Tandem Repeat analysis (MLVA) using 16 Variable Number of Tandem Repeat (VNTR) loci was tested for its ability to discriminate 251 *S. aureus* (MSSA and MRSA) isolates of various clonal complexes obtained from different human, animal and food sources. In total, 155 MLVA types were identified with 21 of them being detected among the 56 CC398 isolates included in this study. Moreover, the MLVA assay was also able to occasionally further differentiate CC398 isolates that shared the same *spa* type.

In conclusion, the results of this Ph.D. project provide not only information on the characteristics of methicillin-resistant staphylococci from bovine mastitis, but also provide guidance in routine diagnostics for the correct assessment of methicillin resistance among CoNS from cases of bovine mastitis and the interpretation of cefoperazone susceptibility testing data of bovine mastitis pathogens. Moreover, the detection of novel resistance genes and their localization on mobile genetic elements demonstrate the need of further research and monitoring of the resistance situation among MRSA CC398.

Chapter 16

Zusammenfassung

Zusammenfassung

Andrea Theresia Feßler (2012)

Vergleichende molekulare Analyse methicillinresistenter *Staphylococcus aureus*- und koagulasenegativer *Staphylococcus*-Isolate aus Mastitisfällen von Milchkühen

In den letzten Jahren war Methicillinresistenz bei Staphylokokken von Menschen und Tieren das Thema vieler Studien. Zu Beginn dieses Ph.D.-Projektes waren jedoch nur sehr wenige Daten zur Methicillinresistenz bei Staphylokokken aus Rindermastitiden verfügbar. Die Ziele der vorliegenden Studie waren eine umfassende Charakterisierung von bovinen methicillinresistenten *Staphylococcus aureus* (MRSA) und methicillinresistenten koagulasenegativen Staphylokokken (MRKoNS) aus Mastitisfällen sowie der Vergleich der MRSA CC398-Isolate von Rindern mit entsprechenden Isolaten anderer Herkunft.

Die genetische Verwandtschaft und die Resistenzeigenschaften von 25 bovinen und zwei humanen MRSA-Isolaten aus 17 Milchviehbetrieben in Deutschland wurden untersucht. Alle Isolate gehörten zum klonalen Komplex (CC) 398. In der ApaI-Makrorestriktionsanalyse dieser Isolate zeigten sich neun Hauptmuster. Drei *spa*- (t011, t034 und t2576), zwei SCC*mec*- (IV, V, ein Isolat nicht typisierbar) und fünf *dru*-Typen (dt6j, dt10a, dt10q, dt11a, dt11ab) wurden nachgewiesen. Die Empfindlichkeitstestung ergab zehn Resistenzmuster. Neben dem *mecA*-Gen, das Methicillinresistenz vermittelt, wurden die Gene *blaZ* (Penicilline), *tet*(K), *tet*(L), *tet*(M) (Tetrazykline), *erm*(A), *erm*(B), *erm*(C), *erm*(T) (Makrolide, Lincosamide, Streptogramin B), *aacA-aphD*, *aphA3*, *aadD* (Aminoglykoside), *spc* (Spectinomycin), *fexA* (Phenicole), *dfrK* (Trimethoprim), *vga*(A) und *vga*(C) (Pleuromutiline, Lincosamide, Streptogramin A) nachgewiesen. Eine ähnliche Situation zeigte sich für ein Testkollektiv von 32 MRSA-Isolate aus Geflügelfleisch/-produkten, das 28 CC398-Isolate mit hoher Variabilität hinsichtlich der Resistenzphäno- und genotypen beinhaltete.

Um die Situation auf Betriebsebene zu untersuchen, wurden 125 MRSA CC398-Isolate von verschiedenen Tierarten und Menschen aus 26 niederländischen Milchviehbetrieben charakterisiert. Insgesamt wurden acht *spa*-Typen (t011, t034, t108, t567, t1184, t1451, t2287, t3934), zwei SCC*mec*-Typen (IV, V sowie 10 nicht typisierbare Isolate), 12 Resistenzmuster und zahlreiche ApaI-Makrorestriktions-muster nachgewiesen. In den einzelnen Betrieben zeigten sich unterschiedliche Situationen. Während bei einigen Betrieben nicht-unterscheidbare MRSA CC398-Isolate bei Menschen und Tieren nachweisbar waren, deutete das Vorkommen verschiedener MRSA-Typen/Subtypen in anderen Betrieben auf verschiedene Übertragungs-/Eintragswege oder eine Veränderung der MRSA-Isolate im Laufe der Zeit hin.

Da die Grenzwerte zur Beurteilung der Methicillinresistenz bei koagulasenegativen Staphylokokken (KoNS) aus der Humanmedizin stammen, wurden 121 Isolate aus Rindermastitiden, den CLSI-Vorgaben entsprechend mit unterschiedlichen Methoden hinsichtlich der korrekten Beurteilung der Methicillinresistenz getestet. Das *mecA*-Gen wurde bei 15 der 16 resistenten Isolate nachgewiesen. Über Oxacillin-Bouillonmikrodilution wurden 10 *mecA*-negative Isolate mit MHK-Werten von 0,5 und 1 mg/L als methicillinresistent beurteilt, während mittels Oxacillin- und Cefoxitin-Agardiffusion alle bis auf jeweils ein Isolat korrekt eingeteilt wurden. Daher sollten KoNS mit Oxacillin-MHK-Werten von 0,5 und 1 mg/L das *mecA*-Gen oder sein Genprodukt nachgewiesen werden, bevor sie als methicillinresistent klassifiziert werden. Die SCC*mec*-Typisierung der 15 *mecA*-positiven Isolate ergab SCC*mec*-Elemente der Typen III, IV und V, wobei ein Isolat nicht typisierbar war.

Diese KoNS-Isolate wurden auch für eine Studie zur Entwicklung einer Empfehlung für klinische Grenzwerte für Cefoperazone mit verwendet, welche insgesamt 1086 Isolate aus den USA und Deutschland, einschließlich *S. aureus*, KoNS, *Escherichia coli*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* und *Streptococcus uberis*, umfasste. Unter Berücksichtigung der im Rindereuter erreichbaren Cefoperazonkonzentrationen wurden die folgenden Grenzwerte vorgeschlagen: empfindlich [MHK-Werte von ≤ 2 mg/L; Hemmhofdurchmesser von

≥ 27 mm (Staphylokokken/*E. coli*) und ≥ 21 mm (Streptokokken)], intermediär [MHK-Werte von 4 mg/L; Hemmhofdurchmesser von 22-26 mm (Staphylokokken/*E. coli*) und 16-20 mm (Streptokokken)] und resistent [MHK-Werte von ≥ 8 mg/L; Hemmhofdurchmesser von ≤ 21 mm (Staphylokokken/*E. coli*) und ≤ 15 mm (Streptokokken)]. Hinsichtlich des Nachweises der Methicillinresistenz, wurden die MRSA-Isolate korrekt von der empfindlichen Population unterschieden, während die MHK-Werte bei den MRKoNS-Isolaten bezüglich der vorliegenden SCC*mec* Kassetten und/oder *Staphylococcus* spp. variierten.

Der derzeitige Kenntnisstand zu (i) plasmid-vermittelten Resistenzen gegenüber Proteinbiosynthese-Inhibitoren bei Staphylokokken und (ii) neuen und ungewöhnlichen Antibiotika-Resistenzgenen bei „Livestock-associated"-MRSA wurde in zwei Übersichtsartikeln dargestellt. Darüber hinaus wurden Resistenz gegenüber Apramycin sowie kombinierte Resistenz gegenüber Linkosamiden, Pleuromutilinen und Streptogramin A-Antibiotika detailliert untersucht. Bislang waren keine Informationen zur Apramycinresistenz von Staphylokokken verfügbar. Untersuchungen an MRSA-Isolaten von Rindern (n=2), Schweinen (n=4) und Geflügelfleisch (n=1) mit Apramycin-MHKs von ≥ 32 mg/L identifizierten ein plasmidlokalisiertes Gen, *apmA*, das für ein Protein aus 274 Aminosäuren kodiert, welches deutliche Homologie zu Acetyltransferasen aufwies. NsiI-Deletion des *apmA*-Gens zeigte eine 16-32-fache Erniedrigung der Apramycin-MHKs, was bestätigte, dass dieses Gen Apramycinresistenz vermittelt. Darüber hinaus wurde eine *apmA*-Variante auf dem kleinen Plasmid pKKS49 von einem porzinen MRSA CC398-Isolat nachgewiesen. Untersuchungen zur kombinierten Resistenz gegenüber Linkosamiden, Pleuromutilinen und Streptogramin A-Antibiotika identifizierten zusätzlich zu den Genen *vga*(A) und *vga*(C) das erst kürzlich beschriebene Gen *vga*(E) erstmalig bei MRSA CC398 von einer Puten (n=1), Rindern (n=2) und Geflügelfleisch/-produkten (n=6).

Die Untersuchung der genetischen Grundlagen für erhöhte Enrofloxacin-MHK-Werte von ≥ 1 mg/L bei 21 MRSA und 15 MSSA der klonalen Komplexe CC398, CC9 und CC5 von Schweinen, Rindern, Geflügel und Geflügelfleisch/-produkten ergab Mutationen in den entsprechenden Zielgenen *grlA*, *gyrA*, *grlB* und/oder *gyrB* sowie in

der Regulatorregion des Gens *norA*. Unabhängig von ihrer Herkunft zeigten alle CC398-Isolate eine Glu422Asp Substitution in der abgeleiteten GrlB-Sequenz und eine spezifische Kombination von Mutationen in der *norA* Regulatorregion, die bei den CC5- und CC9-Isolaten nicht nachweisbar waren.

Außerdem wurde ein neuer Ansatz für die Multiple Locus Variable Number of Tandem Repeat Analysis (MLVA) unter Verwendung von 16 Variable Number of Tandem Repeats (VNTR) Genorten, hinsichtlich der Fähigkeit getestet, 251 *S. aureus*-Isolate unterschiedlicher klonaler Komplexe von Menschen, Tieren und Lebensmitteln zu unterscheiden,. Insgesamt wurden 155 MLVA Typen nachgewiesen von denen 21 bei den 56 CC398-Isolaten dieser Studie zu finden waren. Darüber hinaus erwies sich der MLVA-Assay in der Lage, mitunter CC398-Isolate, die über den gleichen *spa*-Typ verfügten, weiter zu differenzieren.

Zusammenfassend ist festzustellen, dass die Ergebnisse dieses Ph.D.-Projektes nicht nur einen umfassenden Beitrag zu den Eigenschaften methicillinresistenter Staphylokokken aus Rindermastitiden darstellen. In Teilaspekten des Ph.D.-Projektes wurden auch für die Routinediagnostik wichtige Hinweise zur korrekten Beurteilung der Methicillinresistenz bei KoNS aus boviner Mastitis und zur Interpretation der Ergebnisse der Cefoperazon-Empfindlichkeitstestung von bovinen Mastitiserregern erarbeitet. Darüber hinaus zeigt der Nachweis von neuen Resistenzgenen und deren Lokalisation auf mobilen genetischen Elementen die Notwendigkeit von weiterer Forschung und dem Monitoring der Resistenzsituation bei MRSA CC398.

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