Comparative molecular analysis of *Staphylococcus aureus* from intensive livestock farming with emphasis on LA-MRSA of poultry origin
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   Presence of two different *erm(C)*-carrying plasmids in the same methicillin-resistant *Staphylococcus aureus* CC398 isolate from a broiler farm.
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   5th Symposium on Antimicrobial Resistance in Animals and the Environment (ARAE), 30.06. - 03.07.2013, Gent, Belgium, oral presentation Session X, 039

   The gene *lsa(E)* confers combined resistance to lincosamide-pleuromutilin-streptogramin A in methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*.
   5th Congress of European microbiologists (FEMS), 21. - 25.07.2013, Leipzig, Germany, poster 2409

   Combined resistance to lincosamide-pleuromutilin-streptogramin a encoded by the gene *lsa(E)* in methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*.
   2nd Junior Scientist-Symposium FLI, 21. - 24.08.2013, Jena, Germany, poster
Comparative analysis of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from broiler farms as well as from broiler chickens at slaughter and abattoir workers.
DACH Epidemiologietagung “Veterinärmedizinische Epidemiologie in Klinik und Bestandsmedizin”, 04. - 06.09.2013, Hannover, Germany, poster 8

Rapid analysis of the genetic environment of the novel lincosamide-pleuromutilin-streptogramin A resistance gene *lsa*(E) in methicillin-resistant *Staphylococcus aureus* of human, animal and food origin.
National Symposium on Zoonoses Research, 19. - 20.09.2013, Berlin, Germany, poster A05

Identification of the novel spectinomycin resistance gene *spw* in MRSA and MSSA of human and animal origin.
3rd ASM-ESCMID Conference on Methicillin-resistant Staphylococci in Animals: Veterinary and Public Health Implications, 04. - 07.11.2013, Copenhagen, Denmark, poster 84B

Complete sequence of the multi-resistance plasmid pV7037 from a porcine MRSA including two novel resistance genes.
3rd ASM-ESCMID Conference on Methicillin-resistant Staphylococci in Animals: Veterinary and Public Health Implications, 04. - 07.11.2013, Copenhagen, Denmark, poster 86B

* presenting author
To my 'Opi'
„Es wird lange dauern, bis die Menschheit begriffen hat, dass nicht nur die Völker der Erde ein Volk sind, sondern dass Menschen, Pflanzen und Tiere zusammen ‚Reich Gottes‘ sind und dass das Schicksal des einen Bereichs auch das Schicksal des andern ist.“

Luise Rinser, dt. Schriftstellerin (*1911, †2002)
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<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acids</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>BCO</td>
<td>bacterial chondronecrosis with osteomyelitis</td>
</tr>
<tr>
<td>BfT-GermVet</td>
<td>complementary program of the Federation for Animal Health (Ger. Bundesverband für Tiergesundheit e.V. (Bft)) to GERM-VET</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CA-MRSA</td>
<td>community-associated methicillin-resistant <em>Staphylococcus aureus</em></td>
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<tr>
<td>CC</td>
<td>clonal complex</td>
</tr>
<tr>
<td>Clal</td>
<td>restriction nuclease from <em>Caryophanon latum</em> strain L</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
</tr>
<tr>
<td>CoNS</td>
<td>coagulase-negative <em>Staphylococcus</em></td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dr.</td>
<td>doctor</td>
</tr>
<tr>
<td>dru</td>
<td>direct repeat unit</td>
</tr>
<tr>
<td>dt</td>
<td>dru type</td>
</tr>
<tr>
<td>EcoRI</td>
<td>restriction nuclease from <em>Escherichia coli</em> strain R</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td><em>Enterococcus faecalis</em></td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td><em>Enterococcus faecium</em></td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia (engl. for example)</td>
</tr>
<tr>
<td>egc</td>
<td>enterotoxin gene cluster</td>
</tr>
<tr>
<td>EMEA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>GERM-Vet</td>
<td>German Resistance Monitoring-Veterinary Medicine conducted by the Federal Office of Consumer Protection and Food Safety</td>
</tr>
<tr>
<td>HA-MRSA</td>
<td>hospital-associated methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>Hpal</td>
<td>restriction nuclease from <em>Haemophilus parainfluenzae</em></td>
</tr>
<tr>
<td>IS</td>
<td>insertion sequence</td>
</tr>
<tr>
<td>kb</td>
<td>kilo bases</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>LA-MRSA</td>
<td>livestock-associated methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>mg/L</td>
<td>milligramme per litre</td>
</tr>
<tr>
<td>MLST</td>
<td>multilocus sequence typing</td>
</tr>
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</table>
MLS<sub>a</sub> macrolides/lincosamides/streptogramin B
MRS methicillin-resistant staphylococci
MRSA methicillin-resistant <i>Staphylococcus aureus</i>
MRSP methicillin-resistant <i>Staphylococcus pseudintermedius</i>
MSSA methicillin-susceptible <i>Staphylococcus aureus</i>
n number
ORFs open reading frames
P plasmid
PCR polymerase chain reaction
PBP penicillin-binding protein
PFGE pulsed field gel electrophoresis
Ph.D. Doctor of Philosophy
PVL Panton-Valentine leukocidin
QRDRs quinolone resistance-determining regions
rRNA ribosomal ribonucleic acid
<i>S. aureus</i> <i>Staphylococcus aureus</i>
<i>S. epidermidis</i> <i>Staphylococcus epidermidis</i>
<i>S. hyicus</i> <i>Staphylococcus hyicus</i>
<i>S. sciuri</i> <i>Staphylococcus sciuri</i>
SCCmec staphylococcal cassette chromosome <i>mec</i>
SFP staphylococcal food poisoning
SmaI restriction nuclease from <i>Serratia marcescens</i>
spa staphylococcal protein A
spp. species
ST sequence type
t type
Tn transposon
TOC turkey osteomyelitis complex
tpi triosephosphate isomerase
vs. versus
WGM whole genome mapping
WGS whole genome sequencing
XbaI restriction nuclease from <i>Xanthomonas badrii</i>
YOPi young, old, pregnant and immuno-compromised
≥ greater or equal
< less than
% percent
ϕ phi (abbreviation for single-stranded DNA-phages)
CHAPTER 1  General Introduction

The genus *Staphylococcus* consists currently of more than fifty species and subspecies. Some of these species are predominantly found in humans, whereas others are mainly seen in animals. Staphylococci are facultative anaerobic, Gram-positive, catalase-positive and immotile cocci, which commonly form grape-like clusters (SELBITZ 2007). Some staphylococcal species possess the enzyme coagulase, which enables these staphylococci to convert fibrinogen into fibrin. The resulting fibrin coat hides the staphylococci from the host’s cellular immune response. Based on their ability to produce coagulase, staphylococci are differentiated into three groups: the coagulase-positive [e.g. *Staphylococcus aureus* (S. aureus)], the coagulase-negative and the coagulase-variable staphylococcal species (AARESTRUP and SCHWARZ 2006).

In humans and animals, many staphylococcal species, regardless of their ability to produce coagulase, are commensals, which colonize the skin and also represent an integral component of the physiological microbiota on mucosal surfaces of the body, particularly in the upper respiratory tract, but to a lesser extent also in the alimentary or the genitourinary tract (WERCKENTHIN et al. 2001, AARESTRUP and SCHWARZ 2006). However, most staphylococcal species are considered as facultative pathogens and can also cause severe infections in both humans and animals. As a further possibility, staphylococcal species can also occur as contaminants of food of animal origin (WEESE 2010). The three different terms, colonization, infection and contamination are crucially important to understand the importance of staphylococci. Colonization, sometimes also referred to as carriage, describes the multiplying of bacteria in a host without causing disease. In an infection, the presence of the bacterium results in pathological changes that indicate the manifestation of a disease. Contamination describes the presence of the bacteria on or in a material, a physical body or the natural environment, respectively, yet there is no necessity of bacterial multiplication.

In addition to their wide distribution, staphylococci can easily spread between different animal species, and also between humans and the different animal species. Various transmission routes have been described including direct skin to skin contact, contact with excretions or contact with living and non-living vectors.
In veterinary medicine, the most relevant staphylococcal species are *S. aureus*, especially in livestock (KADLEC et al. 2012a), *Staphylococcus hyicus* in pigs (L’ECUYER 1967) and *Staphylococcus pseudintermedius* in dogs (FITZGERALD 2009). In addition, methicillin-resistant *S. aureus* (MRSA) from livestock is the best described species in animals.

The pathogenicity for each single host depends on the host’s health status, but the virulence and resistance genes carried and expressed by the staphylococcal isolates are also of importance (SELBLITZ 2007). Usually, the intact skin and mucosal surface represent the first physiological, mechanical barrier. The functionally active host immune system as well as the tissue-specific commensal microbiota (“physiological flora”) play an important role as biological defence against such pathogens (TIZARD 2004, PODOLSKY 1999). If this system is out of balance as a consequence of injuries, burns, primary viral or parasitic infections, staphylococci can invade deeper tissue and cause localized or generalized infections (WERCKENTHIN et al. 2001).

Furthermore, a potential higher risk exists for an especially sensitive group of individuals, referred to as “YOPI”. “YOPI” stands for very young, old, pregnant or immuno-compromised and it summarizes the four categories of individuals that do not have a robust immune system and are therefore more prone to clinically infections (SKJERVE 2002).

Staphylococcal infections in both, animals and humans, are commonly treated with antimicrobial agents, most often with β-lactam antibiotics (LI et al. 2007). These antibiotics were initially highly effective against staphylococci, but β-lactamase-producing *S. aureus* isolates emerged in the mid-1940s, and their prevalence increased dramatically within a few years (KIRBY 1944). In 1959, Beecham developed the first β-lactamase-stable β-lactam, namely methicillin, to overcome this resistance mechanism. Only two years later, the first isolates of MRSA were described (JEVONS 1961). Meanwhile, methicillin-resistant staphylococci (MRS) have spread throughout the world and have become a major concern in public health and food surveillance.

MRS exhibit many characteristics, particularly their virulence properties and quorum sensing mechanisms, which enable them to continuously cause a wide variety of serious infections in humans (MOELLERING 2012). In addition, MOELLERING (2012) described that the genetic diversity and ability to acquire
genetic material from other bacteria have enabled MRSA isolates to adapt to changing environmental conditions and to modulate their pathogenicity and antimicrobial resistance. These abilities not only empower them to acquire resistance to various antimicrobial agents, but also to establish asymptomatic carriage.

WERTHEIM and colleagues (2005) described that about 20 % of healthy humans persistently carry *S. aureus* in their nose and are considered to be colonized, another 30 % are intermittent carriers and the remaining 50 % of the healthy human population almost never carry *S. aureus*. Nevertheless, the prevalence of nasal colonization with MRSA is much lower and might probably be lower than 1.5 % (GORWITZ et al. 2008). It is easily comprehensible that nasal carriage of *S. aureus* may represent an additional risk factor for the occurrence of staphylococcal infections.

MRSA is one of the most common causes of bacterial infections, ranging in humans from minor skin infections without the necessity to any treatment to severe diseases. In some instances, fatal infections occur in hospitals following a damage of physiological barriers caused by injury or invasive surgery or in immune-compromised patients (CHAMBERS 2001, DIEKEMA et al. 2001). Especially hospital-associated infections with MRSA isolates are rarely trivial (DELEO and CHAMBERS 2009). Although it was initially exclusively a hospital-associated problem, MRSA was in the early 1990s for the first time also described in patients from Western Australia, who had no contact to hospital environment. Meanwhile, MRSA isolates in the community occur worldwide (CHUA et al. 2011).

Currently, MRSA isolates are subdivided into three major groups: hospital-associated MRSA (HA-MRSA), community-associated MRSA (CA-MRSA) and livestock-associated MRSA (LA-MRSA). HA-MRSA isolates and CA-MRSA isolates differ distinctly from each other (DAVID and DAUM 2010). HA-MRSA isolates show high antimicrobial resistance, low virulence and they are not good colonizers. On the contrary, CA-MRSA isolates exhibit low antimicrobial resistance, high virulence [e.g. the Panton-Valentine leukocidin (PVL)] and they are good colonizers that harbor numerous pathogenicity factors. Moreover, HA-MRSA isolates have mostly been isolated from people who are exposed to the health care system, are older and have one or more comorbid conditions. In contrast, CA-MRSA infections tend to occur in previously healthy, younger patients. While HA-MRSA isolates tend to cause
pneumonia, bacteraemia, and other invasive infections, CA-MRSA isolates have been predominantly associated with deep soft tissue infections such as necrotizing fasciitis or septic thrombophlebitis (NAIMI et al. 2003).

The third type of MRSA isolates has initially been identified in pigs, pig farmers and their families from The Netherlands and France in 2005 and showed a characteristic non-typeability by Smal macrorestriction analysis (ARMAND-LEFEVRE et al. 2005, VOSS et al. 2005). The corresponding isolates, first referred to as non-typeable and later on as LA-MRSA, belong most often to the until then rarely identified sequence type (ST) 398 within the clonal complex (CC) 398 (VOSS et al. 2005). In comparison to HA-MRSA and CA-MRSA isolates, LA-MRSA isolates show variable antimicrobial resistance, low virulence and low host specificity.

1.1 Dissemination of LA-MRSA among Animals and Humans

Since the first reports in 2005, LA-MRSA has been reported in many countries, and has become virtually pandemic (CUNY et al. 2010, VANDERHAEGHEN et al. 2010a, GRAVELAND et al. 2011b, LIM et al. 2012). However, the prevalence varies widely from country to country, e.g. prevalence of LA-MRSA of up to 85 % for pigs, 70 % for farms and 45 % for personnel involved in livestock industry has been reported in the United States and Belgium (SMITH et al. 2009, CROMBÉ et al. 2012). In contrast, LA-MRSA has not yet been found at all in Ireland (HORGAN et al. 2011).

According to a study by CUNY and colleagues (2012), there are indications that the rates of LA-MRSA in some farms adhering to an alternative system may be lower, or LA-MRSA may even be absent. It remains to be seen whether these lower rates are true or an artefact resulting from a different sampling or MRSA isolation methodology. If true, it may be worth investigating whether these alternative farms operate outside the mainstream of pig farming (FLUIT 2012). PLETINCKX and colleagues (2011) found that differences in the year of isolation, the sampling site, the type of farm, the time-point of production (age and location) and the methods used for isolation could explain some of the diverging results seen in the different studies. The role of antimicrobial usage and the type of antibiotic used before sampling may also have an impact on the results (CUNY et al. 2012).
In addition, it has been shown that CC398 is not the only predominant CC in livestock. Particularly in Southeast Asia, such as the mainland China, Hong Kong or Malaysia, LA-MRSA belonging to CC9 appear to be more frequent than CC398 (MONECKE et al. 2011, FLUIT 2012). The affiliation to a CC also depends on the host. For instance, the most widespread and dominant *S. aureus* strains in poultry belong to CC5 (LOWDER et al. 2009).

The spread of LA-MRSA in various countries and also the identification in a number of different food-producing animal species suggests a low host-specificity. Beside pigs, LA-MRSA was identified in cattle, namely veal calves (GRAVELAND et al. 2010), feedlot cattle (WEESSE et al. 2012) and dairy cows (FESSLER et al. 2010, HOLMES et al. 2011). LA-MRSA is also present in poultry, both turkeys and chickens on fattening, laying as well as breeding farms (NEMATI et al. 2008, MULDERS et al. 2010, GEENEN et al. 2013).

In pigs, LA-MRSA is mainly a colonizer, and there are only a few studies describing LA-MRSA being involved in clinical diseases of pigs (VAN DUIJKEREN et al. 2007, KADLEC et al. 2009, MEEMKEN et al. 2010). In cattle, LA-MRSA can be a colonizer, but especially in dairy cows - LA-MRSA often causes mastitis (FESSLER et al. 2010, 2012, VANDERHAEGHEN et al. 2010b). Bovine mastitis is a major problem in dairy industry, affecting animal health and causing high economic losses (JONES and BAILEY 2009). Although antibiotic treatment is an option for individual animals, it is expensive, may favour the development of antimicrobial resistance, and does not prevent the chronic presence of *S. aureus* in the udder tissue (FLUIT 2012). In poultry, LA-MRSA can be a colonizer, but several disease patterns have been associated with them as well, such as comb necrosis, leg lameness because of arthritis or osteomyelitis, and septicaemia (NAKAMURA et al. 1997, MCNAMEE et al. 2000, MONECKE et al. 2013). These diseases may affect a significant proportion of a flock and lead to high economic losses. Moreover, the animals suffer and their health status is strongly affected. Furthermore, LA-MRSA has been isolated from other animals, such as dogs, cats, sheep, horses or rats, which were present on the respective farms or had contact to persons at-risk (NIENHOFF et al. 2009, VAN DE GIESSEN et al. 2009, FESSLER et al. 2012).

The contact to food-producing animals and consequently the increased risk of colonization with LA-MRSA is not only a problem for the aforementioned animals, but
also for humans. It quickly became apparent that LA-MRSA were also able to colonize humans (ARMAND-LEFEVRE et al. 2005, VAN LOO et al. 2007b, VAN CLEEF et al. 2011b) and that persons with occupational contact to livestock, such as farmers, veterinarians or abattoir workers, were especially at risk of being colonized by LA-MRSA (HUIJSDENS et al. 2006, CUNY et al. 2009, MULDERS et al. 2010, HUBER et al. 2011, GARCIA-GRAELLS et al. 2012). This is even true for family members of these persons at-risk, even though it seems to play a minor role in the dissemination of LA-MRSA (CUNY et al. 2009).

Further studies revealed that a short-term occupational exposure to pigs or veal calves on LA-MRSA-positive farms for humans not exposed to livestock on a daily basis frequently results in the acquisition of LA-MRSA. However, the majority of people, who acquire LA-MRSA after short-term occupational exposure, get rid of the strain within 24 hours, if there is no repeated contact (VAN CLEEF et al. 2011a). Therefore, colonization could be additionally differentiated in a real persistent colonization and a transient short-term colonization. The persistence of LA-MRSA carriage in humans is consequently dependent on the duration and the frequency of contact with LA-MRSA-positive animals (VAN DEN BROEK et al. 2009, GRAVELAND et al. 2011a). In addition to colonization, LA-MRSA infections in humans have also been described. They preferentially occur in risk groups and account for only a minor part of the global MRSA infections in humans (WULF et al. 2008, RASIGADE et al. 2010, LOZANO et al. 2011, VAN CLEEF et al. 2011b).

Up to now LA-MRSA has also been isolated from horses (VAN DEN EEDE et al. 2009, WALTHER et al. 2009, SIEBER et al. 2011, VINCZE et al. 2014), cats (WEISS et al. 2013a, VINCZE et al. 2014) and dogs (WITTE et al. 2007, VINCZE et al. 2014), all without known contact to livestock or persons at-risk. Besides their role as colonizer or pathogen in different hosts, LA-MRSA, which colonizes food-producing animals, can contaminate carcasses during slaughter and play a role as contaminant in the subsequent manufacturing process (VANDERLINDE et al. 1999). Thus it is not surprising that LA-MRSA has been detected in food of animal origin, such as pork, beef, veal, milk, poultry meat or poultry meat products. This raises the question, which role MRSA from food-producing animals and food plays as a food-borne pathogen (CHAPTER 7).
Not only the general occurrence of LA-MRSA in a variety of hosts and different countries, but particularly the ability to cause zoonotic infections in humans has raised important questions regarding its origin (FITZGERALD 2012). To trace back the origin of LA-MRSA, PRICE and colleagues (2012) applied whole genome sequencing (WGS) to characterize a diverse collection of MRSA and methicillin-susceptible S. aureus (MSSA) from animals and humans originating from 19 countries and four continents. On the basis of the obtained data, the authors assumed that LA-MRSA originated as a MSSA in humans and then spread to and within livestock. The transfer from humans to livestock was accompanied on the one hand by the loss of phage-carried human virulence genes and on the other hand by the acquisition of methicillin and tetracycline resistance genes.

As WGS is not yet possible for all available isolates, in-depth characterization by molecular methods is a good possibility to characterize such isolates to the strain level and to investigate their further spread, since LA-MRSA is not a homogenous lineage or even a single clone. Various molecular typing methods have been established and are also appropriate to trace back the routes of transmission or to identify the most likely sources of contamination (SCHWARZ et al. 2003, FESSLER et al. 2010, 2011b). The discriminatory power of these methods varies distinctly and can be increased by suitable combinations of the methods (FESSLER et al 2011b). Due to variable specificity and different target structures, the choice of typing methods depends on the objective of a study. Furthermore, some of the methods [e.g. macrorestriction analysis, multi-locus sequence typing (MLST), staphylococcal protein A (spa) typing or DNA microarray analysis] are applicable to both MSSA and MRSA, whereas other methods [e.g. staphylococcal cassette chromosome mec (SCCmec) typing or direct repeat unit (dru) typing] are applicable to all MRS, including MRSA.

1.2 Variety of Resistance and Virulence Properties in LA-MRSA

The antimicrobial resistance status in each Staphylococcus isolate can change under a variety of conditions at any time and place. The isolate can be susceptible, resistant to one or two antimicrobial agents or multi-resistant. By definition, staphylococci are referred to as multi-resistant if they have acquired resistance to
three or more classes of antimicrobial agents (SCHWARZ et al. 2010). Resistance to the respective antimicrobial agents can be due to resistance mediating mutations (KADLEC et al. 2011) or to resistance genes, whose products specify different resistance mechanisms. Basically, three mechanisms can be differentiated: (i) enzymatic inactivation of antimicrobial agents, (ii) decreased intracellular accumulation of antimicrobial agents, or (iii) protection/modification/replacement of the cellular target site(s) of the antimicrobial agents (SCHWARZ et al. 2006).

Thereby, the product of a specific resistance gene can confer resistance to specific members of a class of antimicrobial agents or to the entire class or even to members of different classes of antimicrobial agents (KEHRENBERG et al. 2009). On the other hand, resistance to the same antimicrobial agent can be mediated by different resistance mechanisms and/or be based on different resistance genes (SCHWARZ et al. 2006). It is meanwhile commonly accepted that for many resistance properties, two or three resistance genes accounting for more or less the same resistance property may exist simultaneously in the same LA-MRSA isolate (KADLEC et al. 2009, FESSLER et al. 2010, MONECKE et al. 2013).

Previous work on the resistome of LA-MRSA revealed that staphylococci can act as donors and recipients of resistance genes. As staphylococci live in close contact to other bacteria on the skin or the mucosal surfaces, the exchange of genetic material, such as resistance genes, with a wide variety of bacteria, e.g. *Bacillus* spp., *Enterococcus* spp., *Streptococcus* spp., *Lactococcus* spp. or *Lactobacillus* spp., is likely. As a result, the same or closely related resistance genes are often found in bacteria of different species or genera. This is supported by the integration of several resistance genes into mobile genetic elements such as plasmids or transposons.

These elements are not only spread vertically during the division of the host cell, but can also be transferred horizontally, e.g. under selective pressure imposed by the use of antimicrobial agents. As a matter of fact, plasmids are highly flexible: they can undergo recombinational events, form co-integrates with other plasmids, incorporate transposons or parts thereof and can integrate into the chromosomal DNA. Thus, plasmids play an important role as major vehicles of gene transfer into or from LA-MRSA. As a consequence, the acquisition of novel resistance genes in LA-MRSA is considered a continuous process which results from all kinds of interactions of staphylococci with other bacteria (SCHWARZ et al. 2006, KADLEC et al. 2012a).
This explains, why staphylococci (i) harbour a wide variety of resistance genes that confer resistance to several classes of antimicrobial agents, (ii) may show changes of their resistance status due to the loss or acquisition of resistance genes, and also (iii) carry novel resistance genes or resistance genes previously not known to be present in staphylococci.

1.2.1 Common Resistance Pheno- and Genotypes in LA-MRSA

Staphylococci can carry the genes mecA or mecC which account for methicillin resistance. Those isolates are typically abbreviated as MRS. Unfortunately, this leads to confusion from time to time, because it is often believed the ‘MR’ stands for multi-resistant instead of methicillin-resistant, which leads to believe that MRS are in general multi-resistant. The presence of methicillin resistance should to be confirmed by appropriate diagnostic tests, the use of clinical breakpoints and the detection of the methicillin resistance genes mecA or mecC. For that purpose, the existing clinical breakpoints for oxacillin, which were adopted from human medicine, proved to be largely suitable for the identification of methicillin resistance in staphylococcal isolates of animal origin. Both mec genes code for alternative penicillin-binding proteins (PBP) with a strongly reduced affinity to virtually all β-lactam antibiotics (KATAYAMA et al. 2000, GARCIA-ALVAREZ et al. 2011, SHORE et al. 2011). As a consequence, the Clinical and Laboratory Standards Institute (CLSI) recommends that MRS should be considered resistant to the whole class of β-lactam antibiotics (CLSI 2013).

LA-MRSA has been shown to be often resistant to tetracyclines, macrolides, lincosamides, trimethoprim and/or aminoglycosides (KADLEC et al. 2009, FESSLER et al. 2010, 2011b, MONECKE et al. 2011). A closer look at the respective resistance genes so far identified in LA-MRSA revealed resistance genes (i) which are either known to be present in other staphylococcal species of human and animal origin, (ii) which most likely originate from other Gram-positive bacteria or (iii) which are novel and of unknown origin (KADLEC et al. 2012a).

The first group includes resistance genes, such as the β-lactamase gene blaZ (ROWLAND and DYKE 1990), the tetracycline resistance gene tet(K) (LYON and SKURRAY 1987), the macrolide/lincosamide/streptogramin B (MLS\textsubscript{B}) resistance
genes \textit{erm}(A) (MURPHY et al. 1985), \textit{erm}(B) (WERCKENTHIN et al. 1996a) and \textit{erm}(C) (HORINOUCHI and WEISBLUM 1982), as well as the genes \textit{aacA-aphD} (LANGE et al. 2003) or \textit{aadD} (KADLEC and SCHWARZ 2010b) coding for resistance to gentamicin/tobramycin/kanamycin or kanamycin/neomycin, respectively.

The second group includes resistance genes like the tetracycline resistance gene \textit{tet}(L), which is believed to originate from \textit{Bacillus} spp. (KADLEC and SCHWARZ 2009a), the tetracycline resistance gene \textit{tet}(M) (FLANNAGAN 1994), which has been found in a wide variety of Gram-positive and even Gram-negative bacteria, or the MLS\textsubscript{B} resistance gene \textit{erm}(T), which has been detected in \textit{Streptococcus} spp., \textit{Lactobacillus} spp. and \textit{Enterococcus faecium} (DIPERSIO et al. 2008, EGERVÄRN et al. 2009, KADLEC and SCHWARZ 2010a).

The last group comprises amongst others the resistance genes \textit{vga}(C) encoding an ABC transporter for lincosamide/pleuromutilin/streptogramin A resistance (KADLEC and SCHWARZ 2009b), the trimethoprim resistance gene \textit{dfrK} (KADLEC and SCHWARZ 2009a) and the apramycin resistance gene \textit{apmA} (FESSLER et al. 2011a, KADLEC et al. 2012b).

Some of these resistance genes show a preferred localization. The \textit{mec} genes are exclusively located on the chromosomally located \textit{SCCmec} elements. The \textit{tet}(L) gene is often co-localized with the trimethoprim resistance gene \textit{dfrK} on larger plasmids, which can also harbour additional resistance genes such as \textit{erm}(T) or \textit{aadD} (KADLEC and SCHWARZ 2010a), whereas the \textit{erm}(C) gene is most often located on small multi-copy plasmids (AARESTRUP et al. 2000, LÜTHJE and SCHWARZ 2007b). Furthermore, the resistance gene \textit{erm}(A) and the spectinomycin resistance gene \textit{spc} are parts of the transposon (Tn) 554, which is sometimes extended by the lincosamide/pleuromutilin/streptogramin B resistance gene \textit{vga}(E) to the Tn554-like transposon Tn6133 (SCHWENDENER and PERRETEN 2011).

The co-localization of resistance genes enables not only the co-selection, but also the persistence of different resistance genes under the selective pressure induced by the use of one of the respective antimicrobial agents (KADLEC and SCHWARZ 2009a, 2010a). In combination with multi-copy plasmids and all the different opportunities to acquire a variety of resistance genes, there are virtually no limits to exchange genes within and beyond the Gram-positive resistance gene pool.
1.2.2 Common Virulence Factors in LA-MRSA

However, not only the resistance status, but also the presence of virulence properties is of particular importance for veterinary and human medicine. A wide spectrum of secreted and cell surface-associated virulence factors can be expressed to promote adhesion to the host extracellular matrix components, damage host cells or fight the immune system (FOSTER 2005). However, the majority of these virulence factors was identified in different staphylococcal isolates of human origin.

Previous studies on LA-MRSA showed that these isolates, regardless of their origin, usually do not carry the most relevant virulence genes, such as the PVL genes lukF/S-PV, the toxic shock syndrome toxin 1 gene tst1 as well as the exfoliate toxin genes etA, etB and etD (ARGUDÍN et al. 2011, MONECKE et al. 2011). Only in a few cases, LA-MRSA CC398 of human origin has been reported to be PVL-positive (WELINDER-OLLSON et al. 2008, YU et al. 2008).

The vast majority of LA-MRSA CC398 isolates are also negative for enterotoxin genes, whereas LA-MRSA isolates of CC5 and CC9 usually carry the enterotoxin gene cluster (egc), which comprises the enterotoxin G, I, M, N, O and U genes (seg, sei, selm, seln, selo, selu) (MONECKE et al 2011). Most of these enterotoxins are of particular importance, because they can withstand heating processes and have been associated with a form of gastroenteritis that is manifested clinically by emesis with or without diarrhoea in humans, referred to as staphylococcal food poisoning (SFP). SFP is caused by ingestion of one or more enterotoxins via food and food products that have been contaminated with enterotoxin-positive S. aureus isolates (BALABAN and RASOOLY 2000, DINGES et al. 2000).
CHAPTER 2  Aims of the Ph.D. Thesis

The aims of the present Ph.D. thesis were:

(1) to investigate the genetic relationships, the virulence and the resistance properties of *Staphylococcus aureus* (*S. aureus*) isolates associated with diseases in domestic poultry (CHAPTER 3),

(2) to comparatively characterize livestock-associated methicillin-resistant *S. aureus* (LA-MRSA) from broilers at slaughter and abattoir workers as well as from broilers on farm and farm workers for their genetic relationships, their virulence and resistance properties with particular reference to identify transmission between the different reservoirs (CHAPTERS 4-6),

(3) to summarize the current knowledge about MRSA from food-producing animals and food of animal origin with respect to the role of these organisms to act as food-borne pathogens and to consider the available tools for tracking the spread of these organisms (CHAPTER 7),

(4) to summarize the latest information on resistance genes so far detected in staphylococci from healthy and diseased animals (CHAPTER 8),

(5) to analyze the function of a putative ABC transporter identified in MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates of human origin from Spain (CHAPTER 9),

(6) to investigate the genetic basis of pleuromutilin resistance in LA-MRSA of porcine origin from China with particular reference to the genetic environment of the identified plasmid-borne resistance gene *lsa*(E) (CHAPTER 10),
to analyze the function of a putative spectinomycin resistance gene located in a multi-resistance gene cluster in MRSA and MSSA isolates of human origin from Spain as well as from LA-MRSA isolates of porcine origin from China (CHAPTER 11),

(8) to determine the complete sequence of the multi-resistance plasmid pV7037, isolated from a LA-MRSA isolate of porcine origin, to gain insight into its structure and organization with particular focus on a better understanding of the processes that led to the formation of such a multi-resistance plasmid (CHAPTER 12),

(9) to compare MRSA isolates, that harbour the novel genes lsa(E) and spw, for their genotypic relationships, to gain insight into the genetic environment of these novel genes and to see whether these isolates represent part of the multi-resistance gene clusters (CHAPTER 13),

(10) to determine the plasmid background of the novel spectinomycin resistance gene spd among MRSA and MSSA isolates (CHAPTER 14), and

(11) to analyze fluoroquinolone resistance-associated mutations among S. aureus isolates from poultry and other animals as well as food (CHAPTER 15).
CHAPTER 3  Genotyping of S. aureus from Diseased Poultry

Genotyping of Staphylococcus aureus isolates from diseased poultry

Stefan Monecke, Antje Ruppelt, Sarah Wendlandt, Stefan Schwarz, Peter Slickers, Ralf Ehrich, Sonia Cortez de Jäckel

Veterinary Microbiology (2013) 162: 806-12
CHAPTER 4  Transmission of MRSA on Broiler Farms

Transmission of methicillin-resistant *Staphylococcus aureus* isolates on broiler farms

Sarah Wendlandt\textsuperscript{*}, Kristina Kadlec\textsuperscript{*}, Andrea T. Feßler, Dik Mevius, Alieda van Essen-Zandbergen, Paul D. Hengeveld, Thijs Bosch, Leo Schouls, Stefan Schwarz, Engeline van Duijkeren

Veterinary Microbiology (2013) 167: 632-7

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CHAPTER 5  Two Different \textit{erm}(C)-carrying Plasmids in the same Isolate

Two different \textit{erm}(C)-carrying plasmids in the same methicillin-resistant \textit{Staphylococcus aureus} CC398 isolate from a broiler farm

\textbf{Sarah Wendlandt}, Kristina Kadlec, Andrea T. Feßler, Engeline van Duijkeren, Stefan Schwarz

Veterinary Microbiology (2014) doi: 10.1016/j.vetmic.2014.01.009
CHAPTER 6 Characterization of MRSA from Broilers Chickens at Slaughter and Abattoir Workers

Resistance phenotypes and genotypes of methicillin-resistant *Staphylococcus aureus* isolates from broiler chickens at slaughter and abattoir workers

Sarah Wendlandt, Kristina Kadlec, Andrea T. Feßler, Stefan Monecke, Ralf Ehricht, Arjen W. van de Giessen, Paul D. Hengeveld, Xander Huijsdens, Stefan Schwarz, Engeline van Duijkeren


* both authors contributed equally to this study
CHAPTER 7  MRSA: a Food-Borne Pathogen?

Methicillin-resistant *Staphylococcus aureus*: a food-borne pathogen?

Sarah Wendlandt, Stefan Schwarz, Peter Silley

Annual Review of Food Science and Technology (2013) 4: 117-39
Diversity of Antimicrobial Resistance Genes among Staphylococci of Animal Origin

The diversity of antimicrobial resistance genes among staphylococci of animal origin

Sarah Wendlandt, Andrea T. Feßler, Stefan Monecke, Ralf Ehricht, Stefan Schwarz, Kristina Kadlec

International Journal of Medical Microbiology (2013) 303: 338-49
CHAPTER 9  Novel ABC Transporter Gene \textit{lsa}(E)

The enterococcal ABC transporter gene \textit{lsa}(E) confers combined resistance to lincosamides, pleuromutilins and streptogramin A antibiotics in methicillin-susceptible and methicillin-resistant \textit{Staphylococcus aureus}

Sarah Wendlandt\textsuperscript{*,} Carmen Lozano\textsuperscript{*,} Kristina Kadlec, Elena Gómez-Sanz, Myriam Zarazaga, Carmen Torres, Stefan Schwarz


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CHAPTER 10  New Genetic Environment of the Gene \textit{lsa(E)}

Detection and new genetic environment of the pleuromutilin-lincosamide-streptogramin A resistance gene \textit{lsa(E)} in methicillin-resistant \textit{Staphylococcus aureus} of swine origin

Beibei Li*, Sarah Wendlandt*, Jiannan Yao, Yiqiu Liu, Qing Zhang, Zixue Shi, Jianchao Wei, Donghua Shao, Stefan Schwarz, Shaohui Wang, Zhiyong Ma


* both authors contributed equally to this study
CHAPTER 11  Novel Spectinomycin Resistance Gene spw

Identification of the novel spectinomycin resistance gene spw in methicillin-resistant and methicillin-susceptible Staphylococcus aureus of human and animal origin

Sarah Wendlandt, Beibei Li, Carmen Lozano, Zhiyong Ma, Carmen Torres, Stefan Schwarz

CHAPTER 12  Complete Sequence of Plasmid pV7037

Complete sequence of the multi-resistance plasmid pV7037 from a porcine methicillin-resistant *Staphylococcus aureus*

Sarah Wendlandt*, Beibe Li*, Zhiyong Ma, Stefan Schwarz

Veterinary Microbiology (2013) 166: 650-4

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CHAPTER 13  Enterococcal Multi-Resistance Gene Cluster

Enterococcal multi-resistance gene cluster in methicillin-resistant *Staphylococcus aureus* from various origins and geographical locations

**Sarah Wendlandt**, Jun Li, Jeff Ho, Marc Armengol Porta, Andrea T. Feßler, Yang Wang, Kristina Kadlec, Stefan Monecke, Ralf Ehrlich, Jianzhong Shen, Maureen Boost, Stefan Schwarz

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CHAPTER 14 Novel Spectinomycin Resistance Gene spd

Identification of the novel spectinomycin resistance gene spd in a different plasmid background among methicillin-resistant Staphylococcus aureus CC398 and methicillin-susceptible S. aureus ST433

Sarah Wendlandt, Andrea T. Feßler, Kristina Kadlec, Engeline van Duijkeren, Stefan Schwarz

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

Tomasz Hauschild, Andrea T. Feßler, Carmen Billerbeck, Sarah Wendlandt, Heike Kaspar, Joachim Mankertz, Stefan Schwarz, Kristina Kadlec

CHAPTER 16 General Discussion

As a consequence of the increase in world population and the growing global demand for meat, animal husbandry constantly faces new challenges. The performance ability and the flock sizes have to increase steadily. Nevertheless, the health and welfare of animals should rank first. In some countries with high population growth, such as China, the intensive animal husbandry enlarges considerably and the primary goal is the maximum increase of the generated output. In other countries, such as in most European countries, the intensive animal husbandry, that has evolved in the course of structural changing, is being critically judged by the consumers. The livestock-friendly husbandry, which ensures the well-being of animals and their performance, is generally seen positive. Nevertheless, it is discussed controversially in public, especially with regard to the higher costs, the food safety, the environmental effects and public health in general.

The high animal density is a reservoir for pathogens, which can cause infectious diseases. Bacterial infections can be treated with antibiotics, but each use of antibiotics may lead to antimicrobial resistance. Subsequently, these drugs may no longer be effective in people or animals suffering from infections with antimicrobial resistant bacteria. Both, the intensive animal husbandry by itself and the food chain, can serve as possible transmission routes of resistant organisms from food-producing animals or food thereof to humans. Thus, antimicrobial resistance in bacteria from livestock is definitely not only of veterinary interest, but also an issue for human medicine and public health.

16.1 General Considerations

Methicillin-resistant Staphylococcus aureus (MRSA) is an important facultative pathogen which is known to be present in various food-producing animals. MRSA can exhibit resistance to a broad range of antimicrobial agents (LYON and SKURRAY 1987, WERCKENTHIN et al. 2001, JENSEN and LYON 2009). During recent years, MRSA, especially MRSA of the clonal complex (CC) 398, has gained particular attention as colonizer and - more rarely - as causative agent of infections in
food-producing animals (VOSS et al. 2005, WITTE et al. 2007, LEONARD and MARKEY 2008, CUNY et al. 2010). Due to its initial appearance in livestock and in people with occupational contact to livestock, this new MRSA variant was referred to as livestock-associated (LA)-MRSA. Several studies identified LA-MRSA isolates from pig, veal calf and dairy farms (KADLEC et al. 2009, FESSLER et al. 2010, 2012, GRAVELAND et al. 2010) and LA-MRSA has been shown to readily cross species barriers and also colonize and infect humans. In particular, veterinarians, farmers and other persons with exposure to livestock are at risk (CUNY et al. 2009, DENIS et al. 2009, GARCIA-GRAELLS et al. 2012, KÖCK et al. 2013).

Although poultry plays a major role in intensive animal husbandry, there are only limited studies available on LA-MRSA from poultry and also from food of poultry origin intended for human consumption (NEMATI et al. 2008, FESSLER et al. 2011b). Among all food-producing animal species, poultry - especially broilers and turkeys - have the highest number of individuals per herd with 40,000 individuals per flock being the rule rather than the exception. Poultry - in particular broilers - also have the shortest fattening period and based on the high stocking capacity, poultry farms often represent the worst animal husbandry conditions. In spite of or rather due to all aforementioned factors, poultry production is the intensive livestock husbandry with the highest frequency of antibiotic treatments at the moment (VAN RENNINGS et al. 2013). Food of poultry origin represents the second most common source of meat worldwide and simultaneously the cheapest supply of meat (MAENNEL 2013). During recent years, poultry meat has become more popular, particularly amongst consumers from industrialized countries. According to the Federal Association of German Meat Industry, the consumption of poultry meat in Germany increased from 16.7 kilograms (kg) per capita in 2006 to 18.5 kg per capita in 2012 (http://www.bvdf.de). Based on the biological value of poultry protein, the low fat rate and the easy digestibility, food of poultry origin is often consumed by young, old, pregnant and immuno-compromised persons (YOPI’s).

Based on the aforementioned facts, it is of particular importance to comprehensively characterize LA-MRSA isolates of poultry origin (i) to identify the genomic relationships of the isolates present, (ii) to determine their antimicrobial resistance and virulence profiles, (iii) to gain insight into the epidemiological situation, and (vi) to determine possible reservoirs and transmission routes of LA-MRSA of poultry origin and food thereof.
16.2 Genotyping of *S. aureus* from Diseased Poultry

*Staphylococcus aureus* (*S. aureus*) is both a common inhabitant of the skin and mucous membranes of poultry with an ubiquitous presence in the environments, where poultry are hatched, reared or processed, but also a cause of clinically important infections in poultry (SKEELES 1997). The disease conditions vary with the site, the route and the time point of inoculation during the life cycle of poultry. *S. aureus* is the most common pathogen recovered from leg and joint infections in commercial poultry flocks, which is most often associated with bacterial chondronecrosis combined with osteomyelitis (BCO) of the proximal end of the femur and tibiotarsus (MCNAMEE et al. 1998, ALFONSO and BARNES 2006). In turkeys, BCO is additionally associated with a green staining of the liver and thus is referred to as turkey osteomyelitis complex (TOC) (MUTALIB et al 1983, HUFF et al 2000). These diseases are significant in poultry flocks, because the lameness compromises the welfare of poultry and causes considerable economic losses (MCNAMEE and SMYTH 2000, LOWDER and FITZGERALD 2010). Further common staphylococcal infections are umbilical infections or infections of the yolk sac in chicks or turkey poulets, comb necrosis, and, if the bacteria invade the bloodstream, septicaemia (NAKAMURA et al. 1997, SMYTH and MCNAMEE 2008).

In addition to egg or hatchery contamination, predisposing factors to become infected with *S. aureus* include wounds as a result of minor surgical procedures or fighting/cannibalism, immunosuppression based on virus infections or parasite infestations, chronic stress, bad husbandry conditions, e.g. overcrowding (JUNGHERR and PLASTRIDGE 1941, MCCULLAGH et al. 1998, MCNAMEE and SMYTH 2000). Staphylococcosis and many other infections in poultry can be successfully treated with antimicrobial agents, including penicillin, erythromycin and tetracyclines, but antimicrobial resistance is also common (AARESTRUP et al 2000, TANNER 2000).

To gain insight into the resistance properties and into the whole genomic diversity of *S. aureus* isolates associated with diseases in poultry, 51 MRSA or methicillin-susceptible *S. aureus* (MSSA) isolates from clinically diseased chickens and 80 MRSA or MSSA isolates from clinically diseased turkeys were investigated in the first study (CHAPTER 3). All these isolates originated from routine diagnostic
samples at the German Poultry Clinic and Laboratory Dr. Pöppel, Delbrück and were obtained from necropsy material from cases of invasive infections of the joints, liver, heart or lungs.

The molecular typing of the S. aureus isolates revealed significant differences between chickens and turkeys and - especially in turkeys - a high percentage of MRSA was seen. In turkey husbandry, 21.2 % (17 out of 80) were MRSA, whereas the methicillin resistance rate among the chicken S. aureus isolates was only 9.8 % (five out of 51). The most common CC among turkey isolates was CC398. This includes not only the recently emerging livestock associated CC398-MRSA with the staphylococcal cassette chromosome mec (SCCmec) type V (-V) isolates, but also CC398-MRSA isolates with other SCCmec types and CC398-MSSA isolates. Among the chicken isolates, all five MRSA as well as two MSSA isolates belonged to CC398. All CC398-MSSA isolates from turkeys (n=57) showed resistance to penicillins, tetracyclines, and about half of them also to macrolides/lincosamides/streptogramin B (MLS\textsubscript{B}), whereas only one of the two CC398-MSSA isolates from chickens was resistant to penicillins, tetracyclines and MLS\textsubscript{B}. All these observations are in accordance with the results of a recent study by ARGUDÍN and colleagues (2013), where 30 out of 34 isolates from diseased turkeys in Brittany (France) were identified as CC398-MSSA isolates with similar expanded resistance patterns.

The characterization of the 16 CC398-MRSA isolates showed two different staphylococcal protein A (spa) types, seven different direct repeat unit (dru) types, a high variability in their resistance pheno- (n=13) and genotypes (n=16) as well as the lack of major staphylococcal virulence factors. These results revealed striking similarities to CC398-MRSA isolates from diseased pigs (KADLEC et al. 2009), or dairy cattle suffering from bovine mastitis (FESSLER et al. 2010). Consequently, it seems that CC398-MRSA isolates with similar characteristics are associated with a number of different diseases in different food-producing animal species, and their relative homogeneity suggests a more recent expansion. In particular, isolates of CC398-MRSA-V, which was also the common lineage among the MRSA isolates in our study, appear to be very common and widespread and have been reported from most parts of the world in a variety of different host species (WULF et al. 2006, DE NEELING et al. 2007, MONECKE et al. 2007, NEMATI et al. 2008, VAN DUIJKEREN et al. 2008, KRZIWANEK et al. 2009, LOZANO et al. 2009, NIENHOFF et al. 2009,

Two remaining turkey isolates belonged to CC9-MRSA with SCCmec type IV (-IV). The CC9 lineage appears to be livestock-associated in a similar way as CC398 (MONECKE et al. 2011) and CC9-MRSA-IV isolates were also identified in chicken meat products from Germany and The Netherlands (FESSLER et al. 2011) and in chicken isolates as well as chicken meat products from Hong Kong (HO et al. 2012, BOOST et al. 2013).

In contrast to the most frequently detected CC398-MSSA isolates in turkeys, CC5-MSSA isolates were the most frequently isolated lineage in chickens (44 out of 51) in our study (CHAPTER 3). Only four MRSA isolates and four MSSA isolates from turkeys also belonged to CC5. This observation corresponds closely to the data published by LOWDER and colleagues (2009), indicating that CC5 is also a major S. aureus lineage in poultry. This is also in accordance with the results of HASMAN and colleagues (2010), who identified 96 % of their isolates as CC5-MSSA. All methicillin-resistant isolates from turkeys were assigned to CC5-MRSA with SCCmec type III (-III); this MRSA type has previously been isolated from Korean chicken meat samples (KWON et al. 2006) and German turkey meat products (FESSLER et al. 2011), as well as from ill humans in a hospital in the South African province KwaZulu-Natal (SHITTU et al. 2009). The susceptibility testing revealed that all four CC5-MRSA isolates of turkey origin were, in addition to their β-lactam resistance, resistant to tetracyclines, MLSB antibiotics, spectinomycin and enrofloxacin and all but one CC5-MSSA isolates of turkey origin showed at least resistance to penicillins, tetracyclines and MLSB antibiotics. Among the CC5-MSSA isolates from chickens, only single isolates showed resistance to penicillins, tetracyclines and MLSB antibiotics. In contrast to the CC398 isolates, all CC9 and CC5 isolates harboured the enterotoxin gene cluster (egc), which comprises the enterotoxin G, I, M, N, O and U genes.

Two additional lineages were found in turkeys, a single CC7-MSSA isolate and a single CC15-MSSA isolate. Both lineages are abundant among healthy human carriers (MONECKE et al. 2009), so that a transmission from farmers to turkeys can be assumed.

The comparison of our results from both animal species revealed two major differences. First of all, the MRSA rate among the turkey isolates was more than twice as high as among the chicken isolates and most of the turkey isolates proved to
be resistant to a higher number of different classes of antimicrobial agents than the chicken isolates. One possible cause for these observations is that turkeys have a higher risk to be infected by \textit{S. aureus} due to their longer fattening period, which increases the possibility of antimicrobial usage. Nevertheless, further work is needed to determine the true prevalence of MRSA among \textit{S. aureus} isolates from poultry. Secondly, the most frequently detected lineage differed between the animal species. In turkeys, predominantly CC398-MSSA isolates were detected, whereas in chickens CC5-MSSA isolates were more frequent. This raises the question, what the evolutionary origin of these lineages is.

Due to intensive research, a lot about the epidemiology and evolution of \textit{S. aureus} isolates that belong to CC398, especially CC398-MRSA isolates, was ascertained. These isolates referred to as “livestock-associated” (KÖCK et al. 2010, LINDSAY 2010, MONECKE et al. 2011) are widespread in various hosts, including humans, and in most parts of the world. WGS results lead to the understanding that \textit{S. aureus} CC398 originated from a human MSSA lineage, which has subsequently adapted to livestock (PRICE et al. 2012). The transfer of this CC was accompanied not only by the frequent acquisition of methicillin and tetracycline resistance but also by the loss of phage Φ3 and the acquisition of phages Φ2 and Φ6 (PRICE et al. 2012). CC398-MSSA isolates are especially prevalent among pigs in Europe (HASMAN et al. 2010), and as shown in our study also in turkeys. But only based on the high dissemination among pigs and turkeys, it can hardly be determined which - if any - of the species was the first non-human adapted host for the CC398 lineage. However, the common presence of genes from β-haemolysin-converting phages (\textit{sea}, \textit{sak}) in CC398-MSSA isolates of turkey origin as detected in a study by PRICE and colleagues (2012) as well as in our study indicates a closer relationship of human isolates to the majority of CC398-MSSA isolates from turkeys than to those from pigs. Thus, it can be assumed that the human MSSA lineage first spread to turkeys.

CC5 is a common and widespread CC, which comprises a large number of different \textit{S. aureus} isolates among humans, including hospital-associated (HA)-MRSA and community-associated (CA)-MRSA, with a pandemic spread of some of the clones (MONECKE et al. 2011). In livestock, \textit{S. aureus} CC5 isolates have not been investigated to that extent as the CC398 lineage. A study by SMYTH and
colleagues (2009) was the first report, describing *S. aureus* CC5 isolates from broiler chickens with osteomyelitis on a farm in Northern Ireland. In a further study, LOWDER and colleagues (2009) demonstrated that the CC5 poultry isolates were widespread in a number of countries as well as several continents. They reconstructed a natural origin for poultry-associated *S. aureus* CC5 isolates similar to the CC398 evolution. It was demonstrated that all CC5 isolates can be traced back to a single likely human-to-poultry host-jump, which happened about 40 years ago and which originated in or near to Poland. The CC5 host jump also appears to have been followed by the acquisition of avian-niche-specific genes and partial loss of human-niche-specific genes (LOWDER and FITZGERALD 2010).

Further analyses of such host jumps will be important to predict if animals represent a reservoir for the emergence of new virulent clones affecting humans, and conversely if human-to-animal host switches may represent a significant threat to food safety and animal health.

### 16.3 Comparative Characterization of MRSA from Broilers on Farms and at Slaughter as well as from the Respective Workers

Intensive research on LA-MRSA quickly revealed that LA-MRSA was also able to colonize humans (ARMAND-LEFEVRE et al. 2005, VAN LOO et al. 2007b, VAN CLEEF et al. 2011b) and it became apparent that humans with occupational contact to livestock, such as farmers, veterinarians, abattoir workers as well as the respective family members, were especially at risk of being colonized by LA-MRSA (HUIJSDENS et al. 2006, CUNY et al. 2009, HUBER et al. 2011, GARCIA-GRAELLS et al. 2012). Due to this, the prevalence of LA-MRSA on food producing animal farms has been extensively examined in several studies.

GEENEN and colleagues (2013) estimated the prevalence of MRSA-positive broiler farms as well as the prevalence of MRSA carriage in broiler farmers, their family members and employees, and identified risk factors for LA-MRSA. Four of the 50 Dutch broiler farms were tested MRSA-positive, which corresponds to a prevalence of 8.0 %. Furthermore, 66.7 % of people living and/or working on these positive farms were also MRSA-positive, which corresponds to an overall prevalence
of LA-MRSA of 5.5 % in people living and/or working on poultry farms, in contrast to the prevalence of < 0.1 % in the general human population of The Netherlands (BODE et al. 2011). However, the prevalence of LA-MRSA in family members other than the partner (children, parents, siblings) was 0.0 %, which indicates that living on a LA-MRSA-positive farm is not a key factor for becoming a LA-MRSA carrier.

Based on the fact that the intensity of animal contact was found to be an important risk factor for LA-MRSA carriage (VAN DEN BROEK et al. 2009, GRAVELAND et al. 2010), it is speculated that the difference in prevalence could be explained by the difference in time of exposure to LA-MRSA in the poultry houses. This is shown by the fact that farmers and their partners spend significantly more time in the broiler house compared to other family members (GEENEN et al 2013). GEENEN and colleagues (2013) also found a high percentage (nearly 30 %) of LA-MRSA-positive environmental samples in the farm residences on the LA-MRSA-positive farms, whereas none of the samples on LA-MRSA-negative farms was found positive. This finding illustrates the risk to be LA-MRSA-positive as a family member.

In comparison to pig farms (68 - 71 % positive farms, 14 % positive people) or veal calf farms (88 % positive farms, 16 % positive people), the prevalence of LA-MRSA positive broiler farms (8 %) and of people living and/or working on broiler farms (5.5 %) appears to be much lower (BROENS et al. 2011a, c, GRAVELAND et al. 2010). A possible explanation for the reduced risk of LA-MRSA introduction and persistence on broiler farms compared to pig or veal calf farms could be the short duration of the production cycles in broiler farming and the use of an all-in/all-out system. The difference in the prevalence between the respective farmers might be correlated with the corresponding difference in the prevalence of LA-MRSA-positive farms (GEENEN et al. 2013).

A second study by MULDERS and colleagues (2010) estimated the prevalence of LA-MRSA in 40 Dutch broiler flocks from 40 different Dutch broiler farms at the time of delivery at six different slaughterhouses. Furthermore, they (i) determined the degree of MRSA contamination in the different compartments of the slaughterhouse at the beginning and the end of a working day in order to gain insight into routes of transmission and risks of infection for personnel working at the slaughterhouse, (ii) estimated the risk of MRSA carriage in personnel, and (iii) gained
insight into the transmission mechanism of MRSA to humans at broiler slaughterhouses.

Of the slaughter flocks, 35 % were LA-MRSA-positive, based on the presence of at least one positive animal in the specific flock and/or positive transport crate. This prevalence appears to be higher than the 8 % found in the study performed in Dutch broiler farms (GEENEN et al. 2013). A possible explanation for the higher prevalence at the slaughterhouse is the transmission of LA-MRSA during the transports as well as within the slaughterhouses, as it was shown earlier for slaughter pigs (BROENS et al 2011b). In total, 6.9 % of the broilers were LA-MRSA-positive upon their arrival at the slaughterhouse. Of the abattoir workers, 5.6 % were LA-MRSA-positive, which is comparable to the presence of LA-MRSA in broiler farmers, but distinctly increased compared to the general Dutch population (< 0.1 %) (BODE et al. 2011).

However, there are differences regarding the risk rate for LA-MRSA positivity between the different compartments in the slaughterhouses and also between different stunning methods. The risk is significantly larger for personnel having contact with live birds (5.2 %), especially for the workers who hang the live broilers on the slaughter line (20 %), compared to those working only with dead birds (1.9 %). The conventional electric stunning conferred a significantly higher risk of LA-MRSA carriage for abattoir workers than the CO\textsubscript{2} stunning (9.7 % vs. 2.0 %). A possible explanation for this is that when electric stunning is used, birds are placed on the slaughter line alive prior to stunning, which might cause more dust in the environment as a result of the birds flapping their wings. If broilers are stunned with CO\textsubscript{2}, they are hanged only after stunning and wing flapping does not occur (MULDERS et al. 2010).

Furthermore, a spread of LA-MRSA contamination along the slaughter line and in the different compartments of the slaughterhouses can be determined. At the beginning of the production day, the LA-MRSA contamination was relatively low (8 %), particularly in the ‘clean’ areas (processing, cooling and cutting areas), but during meat processing the LA-MRSA contamination increased up to 35 % at the end of the production day (MULDERS et al. 2010).

These two aforementioned studies (MULDERS et al. 2010, GEENEN et al. 2013) served as the basis to gain further insight into the transmission between animals and humans. In our studies, we comparatively analyzed first, all LA-MRSA
isolates from broilers, the farm environment and persons living/working on the LA-MRSA-positive broiler farms (CHAPTERS 4, 5), and second, all LA-MRSA isolates from broiler chickens and abattoir workers from all slaughterhouses, in which LA-MRSA had been identified in both broiler chickens and abattoir workers (CHAPTER 6), with particular focus on their antimicrobial resistance phenotypes and genotypes as well as molecular characteristics.

The first study included a total of 37 LA-MRSA isolates from four different farms (farms 4, 11, 16, 18), including eleven isolates from broilers, 15 isolates from dust in the broiler houses, six isolates from humans, and five isolates from the farm residences (armchair or remote control) (CHAPTER 4). In the second study a total of 46 LA-MRSA isolates from four LA-MRSA-positive slaughterhouses (slaughterhouses 1, 2, 3, 5) were investigated, which comprised 28 isolates from broilers and 18 isolates from humans (CHAPTER 6).

A comparison of the resistance patterns revealed that all LA-MRSA isolates from our two studies showed resistance to at least three classes of antimicrobial agents, except three (two human and one chicken) LA-MRSA isolates of sequence type (ST) 9 from slaughterhouse 1, which were only resistant to β-lactams and fluoroquinolones. The analysis of the resistance genotypes of all LA-MRSA isolates showed the presence of a wide range of resistance genes known to occur in staphylococci (see CHAPTER 8) in various combinations. These findings are similar to the observations in Belgian poultry studies (NEMATI et al. 2008, PERSOONS et al. 2009) and also to those of LA-MRSA isolates from food of chicken or turkey origin (FESSLER et al. 2011b), from diseased pigs (KADLEC et al. 2009) and from bovine mastitis (FESSLER et al. 2010).

In the majority of cases, the resistance genes detected in the LA-MRSA isolates account for the resistance phenotypes observed, but there were cases, in which no gene for a specific resistance property could be detected. This included one human LA-MRSA CC398 isolate from slaughterhouse 5, which exhibited a high tiamulin minimum inhibitory concentration (MIC), but was negative for all pleuromutilin resistance genes, including the lsa(E) gene, which was recently described (CHAPTERS 9, 10). For a long time, the same was true for a number of LA-MRSA isolates, which had high spectinomycin MICs, but were negative for the spectinomycin resistance gene spc and the most recently described novel spectinomycin resistance gene spw (CHAPTER 11). These included: two
indistinguishable chicken LA-MRSA CC398 isolates and the human CC398 isolate from slaughterhouse 2, a single chicken CC398 isolate from slaughterhouse 1, all LA-MRSA isolates from farm 4, the four dust samples from farm 11, the broiler isolate as well as the two isolates from the farm residence on farm 16, and the two dust samples from broiler house 2 on farm 18. Only recently, the second novel spectinomycin resistance gene spd was detected in all these isolates (see CHAPTER 14).

Further analysis revealed that some resistance genes were physically linked to each other or plasmid- and transposon-located, respectively. In this regard, a surprising observation (CHAPTER 5) was made in a single LA-MRSA CC398 isolate from dust of broiler house 2 on farm 18. The plasmid profiling of this isolate revealed among other plasmids, the presence of two small plasmids.

Transformation experiments showed that both small plasmids, designated pSWS371 and pSWS372, conferred resistance to MLS\(_B\) antibiotics and sequence analysis revealed a constitutively expressed \(\text{erm}(C)\) gene on both plasmids. The constitutive expression is based on a deletion within the translational attenuator of 16 base pairs (bp) and 74 bp in pSWS371 and pSWS372, respectively. The same deletions were already described in naturally occurring plasmids (LODDER et al. 1997, WERCKENTHIN et al. 1999) or laboratory mutants (SCHMITZ et al. 2002, LÜTHJE and SCHWARZ 2007a) that carried constitutively expressed \(\text{erm}(C)\) genes. The two plasmids differed in their sizes (2458 bp vs. 3982 bp), their genes for plasmid replication (\(\text{rep}L\) vs. \(\text{rep}F\)) and plasmid pSWS372 carried an additional \(\text{pre}\) gene for a plasmid recombination protein as well as a \(\text{cop}-6\) gene for a small protein potentially involved in copy number control of the plasmid.

Plasmid pSWS371 showed 99 % nucleotide sequence identity to a number of plasmids of different origins (CHEN et al. 2013) or to a part of a tetracycline-MLS\(_B\) resistance plasmid (HAUSCHILD et al. 2005). In contrast, the whole sequence of plasmid pSWS372 was not found in GenBank. The RepF protein corresponded to those of several other staphylococcal plasmids (HORINOUCHI and WEISBLUM 1982) and the Cop-6 protein was identical to the corresponding Cop protein of plasmid SAP085B of \textit{S. aureus} SK1396 identified in 1965 in Australia (ADA79935.1). However, the Pre protein has a unique sequence and the closest amino acids (aa) identity of 59 % exists to the Pre protein of the aforementioned plasmid SAP085B.
The simultaneous presence of two different small plasmids, which carry the same resistance gene and no additional resistance properties in the same LA-MRSA isolate, is a rare and somewhat surprising observation. Only the simultaneous presence of two or three different \textit{erm} genes in the same LA-MRSA isolate (FESSLER et al. 2011b) and the presence of two different \textit{erm} genes on the same multi-resistance plasmid from a LA-MRSA isolate have been described before (GÓMEZ-SANZ et al. 2013b). Under which circumstances these two plasmids have been acquired can only be assumed. However, it is most likely that they have been acquired at different times. The finding that both plasmids belong to different incompatibility groups as specified by the different \textit{rep} genes (NOVICK 1987) may explain why they can stably coexist in the same LA-MRSA isolate.

Referring to all LA-MRSA isolates from both studies, the DNA microarray analysis revealed that all LA-MRSA ST9 isolates carried the \textit{egc} as well as a single isolate each from an employee on farm 11 and a dust sample from farm 18 carried the enterotoxin D (\textit{entD}) and H (\textit{entH}) genes, respectively. All other LA-MRSA isolates did not carry any enterotoxin genes.

Molecular typing revealed that the majority of all LA-MRSA isolates from both studies belonged to CC398 (n=75) and only eight LA-MRSA isolates from the slaughterhouses were assigned to ST9 (n=6) and ST1453 (n=2), respectively. It should be noted that ST1453 is only a single locus variant of ST398. It has the triosephosphate isomerase (\textit{tpi}) allele 175 in contrast to 26, which occurs in ST398, and was first found in these two isolates by MULDERS and colleagues (2010). All LA-MRSA isolates carried \textit{SCCmec} elements of types IV (n=35) or V (n=48). The most frequently isolated \textit{spa} type was t011 (n=55), followed by t034 (n=12), t108 (n=6), t1430 (n=6), t4652 (n=2) and t3015 (n=2). However, the \textit{spa} type t034 (08-16-02-25-02-25-34-24-25), \textit{spa} type t108 (08-16-02-25-24-25) as well as the rare \textit{spa} type t4652 (08-16-02-25-02-25-34-24), respectively, only differ from the more commonly found \textit{spa} type t011 (08-16-02-25-34-24-25) by the loss of one repeat or/and a duplication of two repeats and hence are in fact closely related. The \textit{spa} type t011 is the predominant \textit{spa} type among CC398 isolates of different origins (KADLEC et al. 2009, FESSLER et al. 2010, 2011b), whereas \textit{spa} type t1430 is specific to ST9. Furthermore, the \textit{spa} type t3015 is uncommon and very rare. The two isolates detected in this study are the only ones with this \textit{spa} type, which have
been recorded in the spa typing database. The dru typing revealed several different dru types with dt11a (n=37) as the most frequent one, which corresponds also closely to the results of several studies about LA-MRSA (FESSLER et al. 2010, 2011b, MONECKE et al. 2013, WAN et al. 2013). The macrorestriction analysis pointed out five or six main pulsed field gel electrophoresis (PFGE) patterns with different variants, respectively.

A farm-by-farm analysis revealed that most of the LA-MRSA isolates from broilers, broiler houses as well as humans living and/or working on the respective farm were closely related or even indistinguishable, which indicates transmission between the different reservoirs. This observation was further confirmed by whole genome mapping (WGM). The analysis of a subset of seven isolates from humans or broilers from three farms by WGM showed that the isolates differed considerably between the farms, showing only 94.0 % similarity, whereas the maps of the isolates within farm 4 (99.6 %), farm 16 (99.4 %) and farm 18 (100 %), respectively, were all considered as indistinguishable. These results emphasized the molecular findings and illustrated that WGM represents a useful tool for the investigation of epidemiological links between LA-MRSA isolates (BOSCH et al. 2013).

On farm 4 an interesting observation was made. The partner of the farmer indicated spending on average 2 hours per day in the broiler houses. This contact time in addition to living together with the farmer seemed to be sufficient for colonization with LA-MRSA, because indistinguishable isolates between the partner, the farmer, and the broilers as well as the dust in the broiler house were identified. On farm 16 very similar LA-MRSA isolates from the farmer, his armchair and his remote control, respectively, were detected. It can be assumed that the farmer transmitted the LA-MRSA isolate to his armchair by his hands, or via contaminated dust on his hair or clothes, or by spreading the organism from colonized sites [e.g. by sneezing]. It should be noted that good hygiene precautions, like changing clothes and taking a shower before (re)-entering the farm residence after working in the barns could partly prevent contamination of the farm residence. In addition, facilities like changing clothes in the broiler houses are important in order to prevent transmission of MRSA between barns, between the barns and farm residence and also between farms. A heterogeneity among the isolates from dust in the broiler house, from an employee as well as from the partner of the farmer on farm 11 and
also from dust in a second broiler house on farm 18 compared to all other LA-MRSA isolates on this farm were detected.

In this regard, it should be noted that MRSA can also survive in the environment for prolonged periods of time (WAGENVOORT et al. 2000). There is a variety of possible transmission routes and numerous potential vectors, such as veterinarians, other persons entering the broiler houses, companion animals, colonized rodents, or other animal species present in the environment. Furthermore, the difference among the isolates from broilers and from humans might be explained by exposure of the humans to broilers in different flocks, another origin in earlier production cycles or by exposure to other sources of MRSA. For example, the partner living on farm 11 indicated that she did not have contact with the broilers on the farm, but that she had contact with pigs at least once a month. Thus, it could be speculated that the LA-MRSA isolate cultured from the partner’s nose originated from the pig contact.

A slaughterhouse-by-slaughterhouse analysis revealed that most of the chicken LA-MRSA isolates from the same flock were closely related or even indistinguishable. This apparent homogeneity suggests an exchange of these isolates between the respective animals either at farm or slaughterhouse level. Especially the agitation of poultry during the loading as well as in the slaughterhouses, which results in extensive flapping of wings causes more dust in the environment and also the close contact of the animals during the transportation, can facilitate the exchange of various LA-MRSA isolates between the animals.

In contrast, the LA-MRSA isolates from the abattoir workers at the respective slaughterhouses showed various typing characteristics. Their apparent heterogeneity might arise from the occupational contact of the abattoir workers with animals from numerous chicken flocks. Working in different compartments of the slaughterhouse might also be a reason for the colonization of the abattoir workers with different LA-MRSA isolates. Nevertheless, some LA-MRSA types revealed similar characteristics compared to the chicken isolates and also to isolates from further abattoir workers.

The fact that indistinguishable LA-MRSA isolates from the broilers as well as the abattoir workers were identified, is a strong indication that transmission of LA-MRSA from broilers to abattoir workers occurs, either directly or indirectly through the working environment. The detection of transmission routes revealed that living and/or
working on LA-MRSA-positive broiler farms as well as the occupational contact with broilers at slaughterhouses is a major risk factor for LA-MRSA carriage compared to average members of society. The identification of indistinguishable LA-MRSA isolates from different origins confirms the variety of transmission routes and has identified different vectors for LA-MRSA transmission. The presence of different LA-MRSA isolates on the same farm and at the same slaughterhouse, respectively, indicates that several transmissions took place or diversification of the LA-MRSA isolates happened over time.

These observations are of particular importance regarding public health and for the development of hospital guidelines for intervention. Although this study focused on food-producing animals, contaminated food of animal origin must not be forgotten, as it poses a risk for the general public. As a consequence, this raises the question: Is LA-MRSA a food-borne pathogen?

16.4 MRSA: a Food-Borne Pathogen?

The role of MRSA as a food-borne pathogen is discussed in CHAPTER 7. It is estimated that several million cases of illness each year throughout the world are caused by the consumption of food contaminated with bacterial pathogens, whereby food safety becomes a global health goal (TODD 1997). The first part of the review “MRSA: a food-borne pathogen?” (CHAPTER 7) considers the available methods to investigate the spread of MRSA among animals and/or humans. Various, mostly molecular, methods have been shown to be suitable, but there are many factors that have an impact on the usefulness, and hence the selection of the most appropriate molecular typing method(s) (ENRIGHT et al. 2000, MURCHAN et al. 2003, KONDO et al. 2007, GOERING et al. 2008, IWG-SCC 2009, VAN WAMEL et al. 2010, MONECKE et al. 2011).

The characteristics of the ideal molecular typing method include without limitation (i) high discriminatory power, (ii) ease of analysis and interpretation of results, (iii) timely results, (iv) low costs and (v) practicability of use (GORSKI and CSORDAS 2009). No method is appropriate for all applications, such as short-
long-term epidemiological analyses, outbreak investigations or phylogenetic studies. In many instances, a single typing method does not fulfill all the criteria, necessitating the use of a suitable combination of methods. The aim of the respective study should dictate which method or combination of methods is the most appropriate to be applied.

The second part of the review (CHAPTER 7) summarizes the current knowledge about MRSA from food of animal origin with respect to the role of these organisms in acting as food-borne pathogens. The presence of MRSA in food of animal origin has been globally investigated, including pork, beef and veal, milk, poultry meat and poultry meat products, fish and also meat from lamb, mutton, rabbit, fowl and wild animals (VAN LOO et al. 2007a, DE BOER et al. 2009, LOZANO et al. 2009, LIM et al. 2010, RHEE and WOO 2010, ARGUDÍN et al. 2011, FESSLER et al. 2011b, HARAN et al. 2012).

It should be noted that all studies differ from each other with regard to (i) the origins and number of different origins included in the studies, (ii) the methods used for identification and characterization, and especially (iii) the percentages of MRSA-positive samples in the sample collections investigated. The resulting data revealed different types of LA-MRSA, CA-MRSA, and even HA-MRSA, which can be present in/on food intended for human consumption in different countries. In addition, enterotoxigenic MRSA has also been detected in a number of food sources (KWON et al. 2005, MOON et al. 2007, NORMANNO et al. 2007, RHEE and WOO 2010, FESSLER et al. 2011b, HARAN et al. 2012), but only one case of food intoxication due to MRSA has been reported so far (JONES et al. 2002).

However, to answer the question, whether meat or other food items contaminated with MRSA are likely to increase the risk of consumers becoming colonized or infected, both the MRSA-related aspects, e.g. the MRSA type and its pathogenic potential for humans, and the consumer-related aspects, e.g. the affiliation to the risk group “YOPI” and the infectious dose, must be taken into account.

There are several possible ways for the emergence of contamination of meat, such as direct contamination of the carcasses of colonized food-producing animal during the slaughter process, contamination by transfer of bacteria from the slaughterhouse environment or from colonized/infected personnel. Contaminated raw
meat of animal origin is considered to be a potential vehicle for the transmission of MRSA through direct contact, through consumption of the meat, or through contaminations of other food by direct or indirect contact with the contaminated meat (http://www.bfr.bund.de/cm/349/mrsa_in_food.pdf). Especially the last pathway can dramatically be reduced by adhering strictly to the rules of kitchen hygiene, such as cleaning hands, cutting boards and knives between the preparation of meat and raw food like salad or fruits (BEUMER and KUSUMANINGRUM 2003).

The contamination pathways are diverse and numerous studies identified MRSA-contaminated food of various origins. However, solely the fact that MRSA is present in/on food of animal origin does not qualify MRSA for being considered a food-borne pathogen. Further studies that follow MRSA-colonized animals from the farm via the slaughterhouse to carcasses and meat products would help to identify whether isolates present on the living animal will really end up in the meat/meat products or whether the isolates in final meat products result from contaminations along the food chain. It would also be helpful to investigate which of the known enterotoxin genes are really associated with clinical symptoms, such as vomiting and/or diarrhea. In addition, harmonization of methods for sampling, detection and quantification of MRSA along the food chain as well as for the characterization of these organisms would facilitate the comparison of different studies and would allow a more accurate assessment of the present situation.

16.5 Diversity of Antimicrobial Resistance Genes among Staphylococci of Animal Origin

In comparison to MRSA from food of animal origin, which was the topic of the first review (CHAPTER 7), the data presented in the second review completed within this Ph.D. project summarizes the latest information on resistance genes so far detected in staphylococci from healthy as well as diseased livestock, wild animals and pets (CHAPTER 8).

Several studies identified more than 40 different resistance genes in staphylococci so far (MURPHY 1985, BRISSON-NOËL and COURVALIN 1986, ROWLAND and DYKE 1989, DERBISE et al. 1996, ALLIGNET and EL SOLH 1997,
SCHWARZ et al. 1998, 2000, 2004, O’NEILL et al. 2007, KADLEC and SCHWARZ 2010a, RAMIREZ and TOLMASKY 2010, GARCÍA-ÁLVAREZ et al. 2011), and during the last decade various articles and book chapters also concentrated on the genetic basis of antimicrobial resistance in staphylococci of various animal origins (LYON and SKURRAY 1987, WERCKENTHIN et al. 2001, AARESTRUP and SCHWARZ 2006, JENSEN and LYON 2009, SCHWARZ et al. 2011, KADLEC and SCHWARZ 2012, KADLEC et al. 2012a). Since different detection methods may give different results, the present review (CHAPTER 8) focuses on those genes for which nucleotide sequence data have been deposited in the databases (VAN HOEK et al. 2011, ROBERTS et al. 2012).

Staphylococci of animal origin harbour a variety of resistance genes and the gene products confer resistance to virtually all classes of antimicrobial agents approved for use in animals. This also includes antimicrobial agents, such as florfenicol or apramycin that are approved to control infections caused by bacteria other than staphylococci. In this regard, it needs to be understood that selective pressure is a driving force in the spread of resistance genes not only among the target bacteria, but also among the commensal microbiota ("physiological microbiota"). Thus, staphylococci - as part of the co-resident commensal microbiota in the respective body compartment - may be under selective pressure by antimicrobial agents that are not primarily used to control staphylococcal infections and then develop/acquire resistance. As a consequence, staphylococci, which live as commensals on the skin or the mucosal surfaces, have also acquired resistance genes that allow their survival in the presence of antimicrobial agents such as florfenicol or apramycin (FESSLER et al. 2011a, WANG et al. 2013).

Furthermore, the fact that staphylococci of animal origin do not live in genetic isolation, but are in close contact with numerous other bacteria of the same animal host, enables not only an exchange of a large number of resistance genes between the different staphylococcal groups coagulase-positive, -variable and -negative staphylococci (CHAPTER 8), but also with other Gram-positive bacteria, such as Bacillus spp., Enterococcus spp., Streptococcus spp. or Lactobacillus spp. (NOBLE et al. 1992, LÜTHJE and SCHWARZ 2007b, KADLEC et al. 2012a, LIU et al. 2012, LÓPEZ et al. 2012, CHAPTERS 9, 11). However, the bacteria, in which the respective genes have developed, often have not been identified yet.

In this regard, it should be noted that the number of detected resistance genes - including novel resistance genes - in the respective species depends on the dimension of research work in this field. With regard to staphylococci, distinctly more work on the resistome of LA-MRSA and methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has been conducted during recent years than on the resistome of coagulase-negative staphylococci (CoNS).

The various resistance genes specify different resistance mechanisms and the respective gene products confer resistance to either specific members within a class of antimicrobial agents, to an entire class of antimicrobial agents or to members of different classes of antimicrobial agents (SCHWARZ et al. 2006, KEHRENBERG et al. 2009). Furthermore, for most resistance properties several resistance genes that confer the same resistance phenotype have been detected in staphylococci of animal origin and staphylococci have been shown to carry two or three of these resistance genes simultaneously (KADLEC et al. 2009, FESSLER et al. 2010, KADLEC and SCHWARZ 2012, WEISS et al. 2013a, CHAPTERS 3, 4, 6).

Although even one of these genes is sufficient to confer the respective resistance property, the simultaneous presence of two or three of these resistance genes may be explained by their acquisition at different times and by their different locations, either in the chromosomal DNA, on transposons or on plasmids that carry additional resistance genes (LYON and SKURRAY 1987, KEHRENBERG et al. 2004, COCHETTI et al. 2008, KADLEC and SCHWARZ 2009b, 2010a, WEISS et al. 2013b, CHAPTER 12). The presence of additional resistance genes allows the acquisition of such multi-resistance plasmids or multi-resistance transposons under the selective pressure imposed by the use of one of the corresponding antimicrobial agents. In this regard, co-selection and persistence of resistance genes in the absence of a direct selective pressure need to be taken into account (SCHWARZ et al. 2011). Thus, measures, such as the ban or the limitation of use of certain antimicrobial agents, do not necessarily result in a decrease or loss of resistance genes and resistance properties.

Mobile genetic elements, in particular plasmids and transposons, are not only of particular importance, because they can carry several resistance genes, but they
also facilitate the exchange of antimicrobial resistance genes between different *Staphylococcus* spp. as well as between staphylococci and other Gram-positive bacteria. The detailed analysis of such multi-resistance plasmids (see CHAPTER 12) or multi-resistance gene clusters (see CHAPTER 10), respectively, expanded our understanding of processes such as co-selection and persistence of resistances in staphylococci.


Plasmids play an important role as major vehicles of horizontal gene transfer via transduction, conjugation and mobilization or transformation between bacteria of the same or different species and genera. Thus they contribute to the dissemination of antimicrobial resistance genes. Several mechanisms exist by which plasmids can undergo inter-plasmid recombinational events, form insertion sequence-mediated co-integrates, incorporate transposons or parts thereof, and integrate - in part or *in toto* - into other plasmids or into the chromosomal DNA. These events allow maintenance and the use of the acquired genetic information in the new host, and can result in structurally novel, (multi)-resistance plasmids, and thereby also play a role in the accumulation of resistance genes.

Although the carriage of a wide variety of resistance genes is usually not essential for the survival of the bacteria under physiological conditions, it may be helpful for the bacteria to survive under specific conditions, namely in the presence of antimicrobial agents (TAYLOR et al. 2004, SCHWARZ et al. 2006, 2011). In addition, the physical linkage and organization of resistance genes in a plasmid-borne multi-resistance gene cluster gives excellent options for persistence, but also for co-selection and co-transfer of all corresponding resistance genes by selective pressure imposed by the use of only one corresponding antimicrobial agent (KADLEC and SCHWARZ 2009b, 2010a, GÓMEZ-SANZ et al. 2013b, WEISS et al. 2013b, CHAPTER 12).
The analysis of plasmid-borne multi-resistance gene clusters in LA-MRSA isolates provides insight into the gene acquisition capacities of these isolates, their role as recipients and donors of resistance genes as well as possible partners for resistance gene exchange processes beyond the genus *Staphylococcus*. In this regard, it is hardly surprising that novel or uncommon resistance genes in LA-MRSA were detected (CHAPTERS 9, 11, 14).

16.6.1 Novel Lincosamide-Pleuromutilin-Streptogramin A Resistance Gene *I*sa*(E)*

Combined resistance to lincosamides, pleuromutilins and streptogramin A antibiotics in staphylococci has been attributed to ABC transporters of the Vga type, including those encoded by the genes *vga*(A) and its variant *vga*(A)*v*, *vga*(C) and *vga*(E) (GENTRY et al. 2008, KADLEC and SCHWARZ 2009b, KADLEC et al. 2010b, SCHWENDENER and PERRETTEN 2011, HAUSCHILD et al. 2012). Moreover, in *Enterococcus faecalis* (*E. faecalis*) and *Streptococcus agalactiae*, the genes *lsa*(A) and *lsa*(C) encode ABC transporters of the Lsa type, which mediate a similar resistance phenotype (SINGH a. MURRAY 2005, MALBRUNY et al. 2011).

A major problem in phenotypic identification of all these genes in staphylococci is that (i) lincosamide resistance is not a true indicator as there are numerous other genes in staphylococci which confer lincosamide resistance, (ii) no breakpoints for pleuromutilins and streptogramin A antibiotics applicable to staphylococci are available, and (iii) streptogramin A (except for the expensive substance virginiamycin M1) and streptogramin B antibiotics are not available separately for susceptibility testing. Based on previous experimental experience (KADLEC et al. 2009, FESSLER et al. 2010, 2011b, CHAPTERS 3, 4, 6), MICs of the pleuromutilin tiamulin of ≥ 16 mg/L were used as an indicator to test the corresponding isolates for *vga* genes.

LOZANO and colleagues (2012) determined the genetic environment of the lincosamide nucleotidyltransferase gene *lnu*(B) in one MRSA ST398 isolate and two MSSA ST9 isolates of human origin and identified a putative ABC transporter immediately upstream of the *lnu*(B) gene in their sequenced ~ 12 kilo base (kb) segment. The MICs of tiamulin and also virginiamycin (a compound consisting of
streptogramin A + streptogramin B moieties) were high for all three staphylococcal isolates, a finding which could not be explained by the resistance genes detected in these isolates. Cloning and transfer of the putative ABC transporter into the recipient strain RN4220, followed by susceptibility testing for pleuromutilins, lincosamides and streptogramin A antibiotics, lead to the identification of the novel lsa(E) gene, which was responsible for conferring resistance to these agents (CHAPTER 9).

The lsa(E) gene was the first lsa gene in staphylococci, which confers this resistance phenotype. In addition LOZANO and colleagues (2012) described a high similarity (98.7 %) for about 8550 bp between the lsa(E)-carrying segment and the sequence of the E. faecalis plasmid pEF418.

This finding is another example for a gene flux between Enterococcus and Staphylococcus and supports the assumption that plasmids may play a role in the inter-genus transfer of resistance genes. Similar observations have previously been made for other plasmid- or transposon-borne resistance genes including the vanA gene cluster, the tetracycline resistance gene tet(L), the trimethoprim resistance gene dfrK and the multi-resistance gene cfr (NOBLE et al. 1992, LIU et al. 2012, LÓPEZ et al. 2012, SHEN et al. 2013). These facts emphasize once more the possibility of resistance gene exchanges beyond the genus Staphylococcus.

PCR analysis of seventy porcine LA-MRSA ST9 isolates from China, which showed the same resistance phenotype and harboured none of the other so far known pleuromutilin resistance genes, revealed that 16 of the 70 LA-MRSA isolates were positive for the lsa(E) gene, including a single isolate, in which the lsa(E) gene was located on plasmid pV7037 (CHAPTERS 10, 12).

A 17.5 kb XbaI fragment of plasmid pV7037 was sequenced and contained, in addition to the lsa(E) gene, 13 other open reading frames (ORFs). Structural analysis identified a region bracketed by the two insertion sequences (IS), IS1216 and IS257. Besides the lsa(E) gene, another four resistance genes, aacA-aphD, erm(B), aadE and Inu(B), were detected (see CHAPTER 16.6.3.1). Comparative analysis of this lsa(E)-containing multi-resistance gene cluster on plasmid pV7037 revealed partial homology to the genetic structures found in the MRSA ST398 and MSSA ST9 isolates of human origin from Spain (CHAPTER 9) on the one hand and to plasmid pEF418 from E. faecalis on the other hand. The multi-resistance gene cluster of plasmid pV7037 was even more expanded as it included another two resistance
genes, *aacA-aphD* and *erm* (B), both of which are commonly found in enterococci (HODEL-CHRISTIAN and MURRAY 1991, SAHM and GILMORE 1995, PORTILLO et al. 2000). Based on these findings, it is likely that this multi-resistance gene cluster has evolved in enterococci.

To investigate the genetic environment of the *lsa* (E) gene in the remaining 15 LA-MRSA isolates, 10 different PCR assays were designed that covered the entire multi-resistance gene cluster located on plasmid pV7037. PCR mapping showed that six of the 15 LA-MRSA isolates carried a large part of the multi-resistance gene cluster without the area that comprised the genes *aacA-aphD* and *erm* (B). The remaining nine LA-MRSA isolates carried the gene *lsa* (E), but did not carry the cluster, which suggested a novel genetic environment of the *lsa* (E) gene in these isolates.

The remaining 54 LA-MRSA isolates, which showed the corresponding phenotype, but were negative for all known *vga* genes and also the novel *lsa* (E) gene, were examined for point mutations in domain V of the 23S rRNA and in the gene for the ribosomal protein L3. However, no mutations could be detected in these isolates, which suggests that further novel or uncommon pleuromutilin resistance genes may be present in these isolates.

### 16.6.2 Novel Spectinomycin Resistance Gene *spw*

A closer look at the two different types of multi-resistance gene clusters of MRSA ST398/MSSA ST9 of human origin from Spain (LOZANO et al. 2012) as well as MRSA ST9 of porcine origin from China (CHAPTERS 9, 10), revealed the presence of a reading frame for a putative spectinomycin resistance gene referred to as *orf3* between the resistance genes *aadE* and *lsa* (E) (CHAPTER 11).

Spectinomycin is an aminocyclitol antibiotic and according to European Medicines Agency (EMEA) documents, it is authorised for treatment of enteric and respiratory infections of cattle, sheep, pigs and poultry (EMEA 2001). Resistance to this compound in staphylococci is most commonly associated with the carriage of the *spc* gene, which encodes a 260 aa spectinomycin 9-O-adenyltransferase (MURPHY 1985) and has already been detected in LA-MRSA from pigs (KADLEC et al. 2009,
OVERESCH et al. 2011), cattle (FESSLER et al. 2010), as well as chickens and turkeys (CHAPTERS 3, 6). The spc gene is part of the transposon (Tn) 554 and as such is found in combination with the Tn554-associated MLSB resistance gene erm(A) (MURPHY et al. 1985).

Detailed analysis of the open reading frame for the putative spectinomycin resistance gene revealed an adenyltransferase protein of 269 aa with an alternative start codon (TTG). Database search identified identical or closely related proteins, occasionally with the annotation of a wrong start codon, in Enterococcus faecium (E. faecium), E. faecalis and Lactobacillus johnsonii. In contrast, the putative protein exhibited only 64.7 % identity to the Tn554-associated Spc protein (MURPHY 1985, MURPHY et al.1985). Cloning and transfer of this putative spectinomycin resistance gene into the recipient strain S. aureus RN4220 followed by susceptibility testing for spectinomycin and streptomycin, identified orf3 as a novel spectinomycin resistance gene, which was then designated spw (CHAPTER 11).

The detection of the spw gene, which is likely to be of enterococcal origin, represents another example of the gene flux between enterococci and staphylococci (see CHAPTER 9).

To see whether the spw gene was present in other staphylococci, a PCR assay was developed and applied to four LA-MRSA CC398 isolates from fresh turkey meat and turkey meat products previously identified as spectinomycin resistant and spc-negative (FESSLER et al. 2011b).

Of the four LA-MRSA isolates, one isolate from a seasoned turkey breast schnitzel was positive in the spw-specific PCR, whereas the remaining three were also negative for spw. This observation points towards the existence of other still unknown spectinomycin resistance genes in staphylococci.

16.6.3 Complete Sequence of the Multi-Resistance Plasmid pV7037

After the finding of the two novel antimicrobial resistance genes lsa(E) and spw as part of a multi-resistance gene cluster on plasmid pV7037 from a porcine LA-MRSA ST9 isolate from China (CHAPTERS 9, 10, 11), the complete sequence of plasmid
pV7037 was determined to gain insight into the structure and organization of this plasmid (CHAPTER 12).

The complete sequence of pV7037 consists of a 17,577 bp XbaI segment, which has already been sequenced and carried the multi-resistance gene cluster (CHAPTER 10), and three additional XbaI fragments, which resulted in a total size of 40,971 bp for plasmid pV7037. It should be noted that an additional XbaI site was found to be located in one of the three fragments. However, the plasmid DNA was not restricted at this additional XbaI site, and it is assumed that the original *S. aureus* isolate SA7037 carries a Dam-like methylation system as known from *Escherichia coli*.

16.6.3.1 Fragment Containing the Multi-Resistance Gene Cluster

The previously sequenced ~ 17.5 kb XbaI fragment contained, beside the *lsa*(E) and *spw* gene, the four antimicrobial resistance genes *aacA-aphD*, *erm*(B), *aadE* and *lnu*(B) (CHAPTER 10). The *aacA-aphD* gene codes for a bifunctional enzyme of 479 aa that shows acetyltransferase and phosphotransferase activity and confers resistance to gentamicin, kanamycin, tobramycin and - when over-expressed - also to amikacin (FERRETTI et al. 1986, ROUCH et al. 1987) and is located on the non-conjugative Tn4001 bracketed by IS256 elements (BYRNE et al. 1989). A closer look at the multi-resistance gene cluster revealed that only an IS256-deficient relic of Tn4001 was present. Such truncated Tn4001 elements, in which the terminal IS256 sequence have been deleted, have also been found on multi-resistance plasmids in human *S. aureus* and in avian CoNS isolates, including *Staphylococcus warneri* and *Staphylococcus sciuri* (*S. sciuri*) (LANGE et al. 2003, CARYL and O’NEILL 2009). While the upstream part of Tn4001 was truncated by the integration of IS1216, the downstream part and also 44 bp of the C-terminus of the *aacA-aphD* gene were deleted by the insertion of a Tn917 relic, which generated an alternative stop codon and thus extended the *aacA-aphD* reading frame by 12 codons. Based on the high gentamicin MICs observed, these modifications had no negative impact on the aminoglycoside resistance mediated by the AacA-AphD protein.

The non-conjugative Tn917 was originally found on plasmid pAD2 in the *E. faecalis* strain DS16 and consists of the rRNA methylase gene *erm*(B) conferring
resistance to MLS\(_b\) antibiotics, the resolvase gene \(tnpR\), and the transposase gene \(tnpA\) flanked by inverted repeats of 38 bp. Moreover, an internal direct repeat of 73 bp, which corresponds to the 73 bp at the left terminus of Tn917, separates the resistance gene region from the resolvase/transposase region (SHAW and CLEWELL 1985). On the 17.5 kb XbaI fragment of plasmid pV7037, only the left-hand segment, including the 73 bp terminal repeat, the \(erm(B)\) gene and 39 bp of the internal 73 bp repeat sequence, were present. Such Tn917 relics have also been identified on plasmids from a \(Staphylococcus lentus\) isolate from a mink and a \(S. aureus\) isolate of bovine origin (WERCKENTHIN et al. 1996a, FESSLER et al. 2011a).

The \(aadE\) gene encodes a 302-aa adenyltransferase, which confers streptomycin resistance, and has mainly been found in canine and feline \(Staphylococcus pseudintermedius\) (BOERLIN et al. 2001, KADLEC et al. 2010a, PERRETTEN et al. 2010, GÓMEZ-SANZ et al. 2011, ZAKOUR et al. 2011), but was also detected in porcine \(Staphylococcus hyicus\) (\(S. hyicus\)) (AARESTRUP and JENSEN 2002) and in \(S. sciuri\) (HAUSCHILD et al. 2007).

The \(lnu(B)\) gene codes for a lincosamide nucleotidyltransferase of 267 aa and was primarily identified in \(Enterococcus\) spp, \(Streptococcus\) spp. and \(Clostridium\) spp., however, Lozano and colleagues (2012) recently described the first report of the \(lnu(B)\) gene in MRSA ST398 isolates of human origin. Both genes, \(aadE\) and \(lnu(B)\), are indistinguishable from those previously found on plasmid pEF418 from \(E. faecalis\).

This multi-resistance gene cluster is bracketed by an IS257 element and an IS1216 element, which might have been involved in both the formation of the multi-resistance gene cluster and its dissemination.

**16.6.3.2 Remaining Fragments of Plasmid pV7037**

Apart from the antimicrobial resistance genes in the multi-resistance gene cluster, it contains a \(tet(L)\) gene, a truncated \(\beta\)-lactamase-harbouring Tn552 as well as a \(cadDX\) operon.

The \(tet(L)\) gene codes for a major facilitator protein of 458 aa and confers resistance to tetracyclines except for minocycline (ROBERTS 2005). The \(tet(L)\) gene
was first identified in various *Bacillus* spp., but up to now it has also been shown in different *Staphylococcus* spp., in which it is often located on plasmids (SCHWARZ et al. 1992, 1998). The tet(L) gene of plasmid pV7037 is indistinguishable from the one previously found on plasmid pKKS2187 from a porcine MRSA ST398 (KADLEC and SCHWARZ 2009a), and cloning, transfer, as well as subsequent susceptibility testing of the tet(L) gene confirmed its functional activity.

The non-conjugative Tn552 carries the following genes: the β-lactamase repressor gene *blaI*, the β-lactamase regulator gene *blaR1*, the β-lactam resistance gene *blaZ*, the resolvase gene *binL*, which is involved in a co-integrate resolution system homologous with those of Tn3 family elements, the transposase genes *tnp* and *orf271*. Furthermore, it carries imperfect inverted repeats at its termini (ROWLAND and DYKE 1989, 1990). Tn552 has been detected on plasmids as well as in the chromosomal DNA in various *Staphylococcus* spp. (LYON and SKURRAY 1987). The Tn552 region of plasmid pV7037 exhibits 98.7 % identity to the corresponding sequence of the original Tn552.

The terminal upstream end of the *blaZ* gene was deleted by the integration of an IS431 element and two frame-shift mutations were detected. The first frame-shift mutation was caused by an additional T in the *blaZ* gene, which led to a premature stop codon. The functional inactivity of *blaZ* was confirmed by a low ampicillin MIC. This observation was also recently described for the multi-resistance plasmid pSWS47 (WEISS et al. 2013b). The second frame-shift mutation was caused by a deleted A, which resulted in a premature stop codon in the β-lactamase regulator gene *blaR1*.

In addition to the antimicrobial resistance genes, plasmid pV7037 also harbours a functional *cadDX* operon, which confers resistance to the heavy metal cadmium. The *cadD* gene encodes for a 205-aa P-type metal efflux ATPase protein and the *cadX* gene for a 115-aa protein that serves as a transcriptional regulator (CRUPPER et al. 1999, CHEN et al. 2008). Despite the fact that cadmium is a highly toxic metal that is neither used in agriculture nor found in the community or in the hospital sector, the presence of cadmium resistance determinants on staphylococcal plasmids is relatively common (HOLDEN et al. 2004, MALACHOWA and DELEO 2010, SCHWARZ et al. 2011, GÓMEZ-SANZ et al. 2013a, b).
Elevated concentrations of cadmium in feedstuffs may occur due to the application of sewage sludge or phosphate fertilizers leading to high levels of cadmium in agricultural soils. Although the accepted maximum levels of cadmium in feedstuffs for livestock are regulated, it cannot be excluded that such intake might be in part responsible for the selection of cadmium resistance determinants in the present bacterial population of pigs. The major route of cadmium intake for non-smoking and not occupationally exposed humans is an ingestion of contaminated food (including food of animal origin) and water (EFSA 2004, ASHRAF 2012, CIOBANU et al. 2012). This exposure might also select for cadmium resistance in transferable elements in the human _S. aureus_ population and thus can easily spread to animals.

Database search for sequence similarity on nucleotide level exhibited 100 % identity of the _cadDX_ operon on plasmid pV7037 to the corresponding sequence of plasmid pUR3912 from a MSSA ST398 isolate from a healthy human in Spain (GÓMEZ-SANZ et al. 2013c).

Additionally, analysis of the complete sequence of plasmid pV7037 focused on two additional putative ORFs, which attracted attention: firstly, the RNA methylase gene, previously identified in the multi-resistance gene cluster and referred to as _orf2_, and secondly, an ABC transporter gene upstream of the _tet(L)_ gene, which showed high similarity on protein level to ABC transporters identified in _Staphylococcus epidermidis_ (S. epidermidis). No defined function has been assigned to the two ORFs and susceptibility testing of the corresponding _S. aureus_ transformants indicated that the two genes obviously do not have a function in antimicrobial resistance.

In addition to the resistance genes, plasmid pV7037 harboured two intact and one truncated _IS431_ elements, one _IS257_ element and _IS1216_ element. While _IS1216_ is mainly found in _Enterococcus_ spp., _IS431_ and _IS257_ have commonly been identified in different _Staphylococcus_ spp. and have been shown to play an important role in the distribution of a variety of antimicrobial resistance genes in staphylococci. Insertion sequences can facilitate integration- and recombination processes in the chromosomal DNA and also on plasmids, and furthermore result in structural rearrangements of plasmids (SIGUIER et al. 2006, SCHWARZ et al. 2011). For example, _IS257_ elements have been assumed to be involved in the integration of the small tetracyclines resistance plasmids into larger plasmids (WERCKENTHIN et al. 2006).
such as the ∼ 40-kb plasmid pKKS2187 from a porcine MRSA ST398 isolate (KADLEC and SCHWARZ 2009a). In addition, plasmid pV7037 harbours further functional genes, which encode proteins required for replication, mobilization and recombination processes.

In conclusion, the complete sequence analysis of plasmid pV7037 confirmed that this plasmid contains a total of seven functionally active genes which mediate resistance to antimicrobial agents, some of which are commonly used in veterinary medicine, while others are classified as critically important in veterinary medicine [e.g. macrolides] and some even represent reserve agents in human medicine [e.g. streptogramins, pleuromutilins]. The use of any of these antimicrobial agents could provide selective pressure for the maintenance and dissemination of the multi-resistance plasmid pV7037. In addition, the emergence of such multidrug resistance plasmids that also carry heavy metal resistance genes such as the cadDX operon, is alarming and may also facilitate their persistence, co-selection and dissemination.

The mosaic structure of plasmid pV7037 strongly suggests that this plasmid has been developed from various parts that have been identified before on other plasmids or transposons of staphylococci, enterococci or other Gram-positive bacteria. Similar mosaic structures were also described in other recently sequenced multi-resistance plasmids. For example, the multi-resistance plasmid pSWS47 from a feline methicillin-resistant S. epidermidis ST5 isolate also carried five functionally active antimicrobial resistance genes, several insertion sequences and was also composed of parts of plasmids and transposons previously described in staphylococci as well as other Gram-positive bacteria (WEISS et al. 2013b).

This finding underlines once more the ability and enormous flexibility of bacteria to exchange genetic material across species and genus boundaries and to generate novel mosaic plasmids with numerous resistance genes from different sources, in order to find ways and means to escape the effects of antimicrobial treatment.
16.7 **Enterococcal Multi-Resistance Gene Cluster**

In recent studies, two different types of an enterococcal multi-resistance gene cluster were identified in MRSA ST398 and MSSA ST9 isolates of human origin from Spain (LOZANO et al. 2012, LI et al. 2014, see CHAPTER 9) as well as on plasmid pV7037 or in part in the chromosomal DNA of MRSA ST9 isolates from swine in China (see CHAPTER 10, 11, 12). Both types harboured the novel resistance genes *lsa*(E) and *spw* along with the resistance genes *aadE* and *lnu*(B), while the cluster identified on plasmid pV7037 also carried the genes *aacA*-aphD and *erm*(B).

During the analysis of LA-MRSA isolates from various origins and geographical locations, several isolates were identified as spectinomycin- and pleuromutilin-resistant, but were negative for the until then known resistance genes, which confer these phenotypes. These included ten LA-MRSA CC9 isolates from frozen or chilled chicken and pig carcasses from five different wet markets and a supermarket in Hong Kong, a single LA-MRSA CC9 isolate from a butcher working at a further wet market and a single MRSA CC398 isolate of a nasally colonized human patient in a German hospital (CHAPTER 13). Meanwhile, the novel resistance genes *lsa*(E) and *spw* were identified in these isolates by specific PCR assays.

Molecular analysis revealed that all LA-MRSA CC9 were closely related. These isolates had the atypical SCCmec type V with *spa* type t899 and *dru* type dt12w, except single isolates with *spa* type t1234 and *dru* type dt12y, dt11by or dt10ca. To see more subtle variations among these isolates, macrorestriction analysis was used, which is considered as the most discriminative and most suitable method for short-term epidemiological analyses. These pattern-based method exposed not only LA-MRSA CC9 isolates with indistinguishable or slightly variable PFGE patterns within the same wet market, but also individual PFGE patterns within and between the different wet markets. Susceptibility testing, DNA microarray analysis as well as further PCR assays revealed five different resistance phenotypes and four different genotypes among the MRSA CC9 isolates and all of which were considered as multi-resistant. In contrast, the single MRSA CC398 isolate belonged to SCCmec type V, *spa* type t011 as well as *dru* type dt11a and was also considered as multi-resistant by its resistance to three or more classes of antimicrobial agents.

Furthermore, ten overlapping PCR assays (see CHAPTER 10) and also whole genome sequencing identified a large part of the pV7037-associated multi-resistance
gene cluster in all twelve MRSA isolates, including the resistance genes \textit{aadE}, \textit{spw}, \textit{lsa}(E) and \textit{Inu}(B). In comparison to all LA-MRSA CC9 isolates, which carried downstream of the \textit{Inu}(B) gene a transposase gene, the single MRSA CC398 isolate from a human patient exhibited at this position two genes coding for a resolvase and a nucleotidytransferase from \textit{E. faecium}, respectively.

In accordance with the majority of the Chinese porcine MRSA CC9 isolates and the Spanish MRSA ST398 and MSSA ST9 isolates of human origin, different transfer experiments suggested that the cluster is located in the chromosomal DNA of all twelve MRSA isolates. All these observations suggest that a multi-resistance gene cluster, which is closely related to the one detected in enterococci of human and porcine origin by LI and colleagues (2013), has obviously acquired by MRSA/MSSA CC9 and CC398 isolates of various origins independently at different occasions in Europe and Asia.

16.8 Novel Spectinomycin Resistance Gene \textit{spd}

During recent years, several survey studies revealed a high frequency of LA-MRSA isolates, which displayed phenotypic resistance to spectinomycin. However, only a few were found to carry the commonly spectinomycin resistance gene \textit{spc} or the novel spectinomycin resistance \textit{spw} (see CHAPTER 11). More recently, a further novel spectinomycin resistance gene, designated \textit{spd}, has been identified on the small plasmid pDJ91S in MRSA ST398 isolates from chicken, horse, rat, cattle, pig and human origin in Belgium (JA\textsc{mrozy} et al. 2014).

To see whether the \textit{spd} gene was responsible for the phenotypic resistance to spectinomycin, the \textit{spd}-specific PCR assay described by JA\textsc{mrozy} and colleagues (2014) was applied to LA-MRSA/MSSA isolates from different human and animal sources outside Belgium (CHAPTER 14). These included four LA-MRSA CC398 isolates from broiler chickens at slaughter and abattoir workers in The Netherlands (see CHAPTER 6), 21 LA-MRSA CC398 isolates from broiler farms, humans and their households in The Netherlands (see CHAPTER 4), four LA-MRSA CC398 isolates from turkey meat and meat products in Germany and Austria (see FESS\textsc{essler} et al. 2011b), a single LA-MRSA CC398 isolate from a diseased pig in the GERM-Vet
program (see KADLEC et al. 2009), and a single LA-MSSA ST433 from a diseased pig in the BfT-GermVet study (see SCHWARZ et al. 2007). All these isolates were positive in the spd-specific PCR, Southern blot hybridization tinging of plasmid profiles from the 31 isolates with a spd-specific gene probe confirmed the presence of a small ~ 3.9 kb plasmid that hybridized with the spd probe.

Electrotransformants harboured the expected small plasmid and sequence analysis of a single plasmid, designated pSWS2889, revealed a complete size of 3898 bp which is 30 bp smaller than plasmid pDJ91S. The major part of the two plasmids, including the Spd protein and a plasmid recombination/mobilization protein, were indistinguishable, while considerable differences were noticed between their Rep proteins. In comparison to the 322 aa Rep protein of plasmid pDJ91S, the 8 aa difference to the 314 aa Rep protein of plasmid pSWS2889 was due to a loss of one tandem copy of a 24 bp segment. The protein alignment of the two Rep Proteins showed an overall identity of only 81.1 %.

Furthermore, the sequence differences in the two rep genes also account for unique restriction sites for the endonucleases HpaI, EcoRI and Clai in pDJ91S which can be used to differentiate between the two similar sized spd-carrying plasmids. Based on this fact, restriction analysis - confirmed by sequence analysis of the rep gene of selected plasmids - revealed that plasmid pSWS2889 was found in 22 MRSA isolates, whereas plasmid pDJ91S was present in the remaining nine isolates.

In conclusion, both plasmid types could be detected in isolates of various origins and geographical locations that date back until 2005 and it was the first identification of the spd gene in a LA-MSSA ST433 isolate. These findings demonstrated that the spd gene can be present on different plasmid types in LA-MRSA/MSSA isolates and is obviously present for longer times than initially thought. Moreover, the emerging diversity of novel spectinomycin resistance genes as well as the frequent detection of these novel genes might suggest a significant selective pressure on LA-MRSA/MSSA isolates and putative high rates of horizontal transfer of the associated plasmids.
16.9 **Fluoroquinolone Resistance-Associated Point Mutations**

Besides resistance genes, mutations in the target genes can also account for antimicrobial resistance (WOODFORD and ELLINGTON 2007). Mutation-mediated resistance is especially common among resistances to synthetic antibacterial agents, such as fluoroquinolones and oxazolidinones (TAKAHASHI et al. 1998, HOOPER 2000, TSIODRAS et al. 2001, WILSON et al. 2003, MEKA et al. 2004).

Enrofloxacin was the first fluoroquinolone approved for veterinary medicine and is still commonly used in veterinary practice to treat infections caused by a wide spectrum of bacterial pathogens, including MSSA and MRSA (MARTINEZ et al. 2006, LIN and DAVIES 2007). Fluoroquinolones interact with two bacterial targets, the related enzymes DNA gyrase and topoisomerase IV, both of which are involved in DNA replication (DRLICA and ZHAO 1997).

The tetramer DNA gyrase is composed of two GyrA and GyrB subunits and is the only bacterial enzyme that introduces negative superhelical twists into the DNA, which is important for the initiation of DNA replication. DNA gyrase also facilitates DNA replication by removing positive superhelical twists that accumulate ahead of the replication fork, or as a result of transcription of certain genes (WANG 1996, HOOPER 1998).

Topoisomerase IV is similarly structured and is composed of two GrlA and GrlB subunits each (FERRERO et al. 1994). It is active in the terminal stages of DNA replication, allowing for the decatenation of interlinked daughter chromosomes so that segregation into daughter cells can occur (ADAMS et al. 1992).

Fluoroquinolones inhibit these two enzymes by stabilizing either the DNA–DNA gyrase complex or the DNA–topoisomerase IV complex, which results in a rapid inhibition of DNA synthesis and eventual cell death (DRLICA a. ZHAO 1997, HOOPER 1998). But it should be noted that fluoroquinolones differ in their relative activities against these enzymes, and topoisomerase IV was found to be the primary target in *S. aureus* (FERRERO et al. 1994). Development of resistance to fluoroquinolones is a stepwise process, resulting from the accumulation of amino-acid substitutions in these two subunits (HAWKEY 2003). Moreover, an increasing number of mutations generally correlates with increasing MICs (RUIZ 2003). Most, but not all, of the mutations fall into defined regions of the genes *gyrA*, *gyrB*, *grlA* and...
grlB, designated the quinolone resistance-determining regions (QRDRs) (TAKAHASHI et al. 1998).

Another important factor in the determination of fluoroquinolone resistance in *S. aureus* is the overexpression of the *norA* gene by mutations in the *norA* promoter region. This gene encodes a multidrug efflux pump that can actively remove the antibacterial agent from the cell (KAATZ et al. 1993, SCHMITZ et al. 1998, TAKAHASHI et al. 1998).

The distribution of fluoroquinolone resistance-associated point mutations was examined in 21 MRSA and 15 MSSA isolates from diseased pigs (MRSA n=8, MSSA n=3), cattle (MRSA n=2), poultry (MRSA n=2, MSSA n=12) and food of poultry origin (MRSA n=9) (CHAPTER 15), which had already been characterized in previous studies (SCHWARZ et al. 2007, KADLEC et al. 2009, FESSLER et al. 2011b). Unfortunately, Clinical and Laboratory Standards Institute (CLSI) approved clinical breakpoints for enrofloxacin applicable to *Staphylococcus* spp. are restricted to staphylococci from cats and dogs (susceptible: ≤0.5 mg/L; intermediate: 1 - 2 mg/L; resistant: ≥ 4 mg/L) and no CLSI-approved clinical breakpoints applicable to staphylococci from pigs, cattle or poultry are currently available (CLSI 2013). Based on the aforementioned breakpoints, the analyzed isolates were selected from five previous isolate collections based on their enrofloxacin MIC of ≥ 1 mg/L.

Twenty-four of the 36 MRSA/MSSA isolates were assigned to CC398, six to CC5 (all of poultry origin or food thereof) and five to CC9. The remaining avian isolate belonged to the novel sequence type ST2269 that has been identified for the first time during the course of this study (CHAPTER 15).

Two or three mutations in the QRDRs of the genes *gyrA*, *gyrB*, *grlA* and/or *grlB* were detected in all isolates, except for two MRSA CC398 isolates from pigs, which showed only a single mutation in the *grlB* gene. Regardless of their origin and their methicillin resistance status, all isolates with enrofloxacin MICs of ≥ 4 mg/L contained the already described GrlA substitution Ser80Phe and one of the GyrA substitutions Ser84Leu (TAKAHASHI et al. 1998) or Ser84Ala; the latter was first reported in staphylococci in our study in the two MRSA isolates of porcine origin (CHAPTER 15).

In contrast, isolates with enrofloxacin MICs of 1 or 2 mg/L showed one of the GrlA substitutions Ser80Phe, Ser80Tyr or Ser80Leu, but lacked the GyrA
substitutions at position 84. 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).

Moreover, except for a single isolate of turkey meat origin, which showed only an exchange in the GyrB protein (Arg517Lys), all CC398 isolates, had an additional Glu422Asp exchange in their GrlB, regardless of their enrofloxacin MIC, the year of isolation, and the source from which the isolate was obtained. A single avian MRSA ST5 isolate also showed a GrlB substitution at position 471. Another interesting observation was the presence of the Ser80Leu and Glu84Asp GrlA exchanges and the GyrA exchange Glu88Asp in the MRSA ST2269 isolate of poultry origin. These substitutions have not been identified so far in *S. aureus*.

Furthermore, two types of point mutations were detected in the *norA* promoter region; all CC398 isolates shared the same type of mutation, whereas the remaining isolates showed the second type, except the single avian MRSA ST2269 isolates for which no *norA*-specific PCR product could be obtained. However, these point mutations affected neither the *norA* promoter sequences nor the *norA*-associated ribosomal binding site.

In summary, it can be stated that increased MICs of enrofloxacin among *S. aureus* isolates from diseased food-producing animals or food of animal origin is mainly mediated by mutations in *grlA* and/or *gyrA*, which corresponds to the findings reported previously in *S. aureus* isolates of different origins (TAKAHASHI et al. 1998). The detection of four novel mutations during this study additionally illustrates the variety and the potential for mutational resistance to emerge.
Results of the first part of this Ph.D. thesis (CHAPTERS 3, 4, 6) demonstrated that livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) isolates showed similar genomic characteristics as well as resistance and virulence properties, even though they were isolated from different origins: from healthy poultry on farms or at slaughter, from dust samples in broiler houses, from humans living and/or working on the poultry farms or from humans working at the poultry slaughterhouse, from the farm residences, or even from clinically diseased poultry collected during necropsy. LA-MRSA from the same farm or flock at slaughter exhibited a high homogeneity indicating a transmission of these bacteria between poultry at farm or flock level. However, two or more LA-MRSA clones were often present in one flock/farm, indicating that either several introductions events took place or a diversification of a specific LA-MRSA isolate occurred over time. In contrast, the LA-MRSA isolates from the abattoir workers were more heterogeneous, which might reflect their occupational contact with animals from numerous chicken flocks and thereby with different LA-MRSA isolates.

There is a tendency to declare livestock-associated *Staphylococcus aureus* synonymous with MRSA of the sequence type (ST) 398. However, as illustrated by the different test populations, multiple STs belonging to different clonal complexes (CCs) are livestock-associated, e.g. CC5 or CC9 in poultry. Moreover, all these CCs represent MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates. In addition, human-associated isolates are also found among livestock, as LA-MRSA CC398 isolates can be found among humans without direct contact to livestock. Nevertheless, among LA-MRSA-/MSSA isolates, CC398 isolates seem to dominate; even so it is not clonal, but consists of a highly variable set of clones.

Results of the second part of this Ph.D. thesis (CHAPTER 7) revealed that there is little evidence for LA-MRSA being a food-borne pathogen. Nevertheless, data have shown that LA-MRSA and also community-associated and hospital-associated MRSA can be present in/on food items intended for human consumption. In order to estimate the risk of consumers becoming colonized or infected, the MRSA-related aspects, e.g. type of MRSA or pathogenic potential, and the consumer-related aspects, e.g. age or health status, must be taken into account.
Despite these aspects, the most effective measure to reduce the risk becoming colonized or infected by MRSA-contaminated food is to adhere strictly to the rules of kitchen hygiene.

Results of the third part of this Ph.D. thesis (CHAPTERS 5, 8-15) illustrated the various opportunities of Staphylococcus spp. from different animals as well as from humans to become resistant by either acquisition of novel resistance genes of unknown origin, acquisition of resistance genes already present in other Gram-positive bacteria, or by developing target gene mutations. Given the hundreds of millions of years of their potential exposure to antibiotics in nature, it is not surprising that staphylococci are prepared to overcome the inhibitory effects of most antimicrobial agents by accepting and also passing on mobile resistance genes present within the Gram-positive gene pool. The knowledge of whether a resistance gene is present on a plasmid or a transposon and whether it is co-located or even physically linked with other resistance genes is important information to predict its further dissemination among staphylococci and other Gram-positive bacteria. Besides their role as human and animal pathogens, the vast majority of the staphylococci represent part of the commensal microbiota ("physiological flora") on the skin and the mucosal surfaces of the upper respiratory and alimentary tract. These commensal staphylococci also play an important role as donors and as recipients of antimicrobial resistance genes. Based on these facts, prudent use of every antimicrobial agent is strongly recommended in both human and veterinary medicine to retain the efficacy of antimicrobial agents for the control of bacterial infections.
CHAPTER 18  Summary

Sarah Wendlandt

Comparative molecular analysis of *Staphylococcus aureus* from intensive livestock farming with emphasis on LA-MRSA of poultry origin

Since a few years *Staphylococcus aureus*, especially livestock-associated methicillin-resistant *S. aureus* (LA-MRSA), has been the focus of many studies, but only limited information on LA-MRSA from poultry origin was available at the beginning of this Ph.D. project. The aims of this study were (i) to comprehensively characterize livestock-associated *S. aureus* of poultry origin, (ii) to answer the question if MRSA is a food-borne pathogen, (iii) to provide an overview on antimicrobial resistance genes in staphylococci, and (iv) to identify novel antimicrobial resistance genes in LA-MRSA isolates.

In the first part of the Ph.D. project, three different test populations were investigated. The first test population included 131 isolates from clinically ill turkeys and chickens (CHAPTER 3). Among the turkey isolates (n=80), the clonal complex (CC) 398 predominated and 21.2 % were LA-MRSA. The majority of chicken isolates (n=51) belonged to CC5 and only 9.8 % were LA-MRSA, however, all were identified as LA-MRSA CC398. In the other two studies 37 LA-MRSA CC398 isolates from four different broiler farms (broilers n=11, broiler houses n=15, farm residences n=5, humans living and/or working on the farms n=6) (CHAPTER 4) and 46 LA-MRSA CC398 and CC9 isolates from broilers at four different slaughterhouses (n=28) and workers at the respective poultry slaughterhouses (n=18) (CHAPTER 6) were investigated in order to explore transmission between different reservoirs. Molecular analysis of all LA-MRSA isolates from the three test populations showed two SCCmec types (IV and V), seven spa types (mainly t011) and twelve dru types (mainly dt11a or dt10q). Susceptibility testing, DNA microarray analysis and/or gene specific PCR assays, respectively, revealed highly variable resistance phenotypes (n=41) and genotypes (n=40). All LA-MRSA isolates (except three LA-MRSA CC9 isolates) were considered as multi-resistant due to their resistance to three or more classes of antimicrobial agents. These results showed that the current dissemination
of LA-MRSA also included chickens and turkeys. The LA-MRSA isolates from the same farm or flock at slaughter exhibited a high homogeneity indicating transmission between poultry at farm or flock level. In contrast, the heterogeneity of the LA-MRSA isolates from the abattoir workers might reflect their occupational contact with animals from numerous poultry flocks.

In addition, a rare observation was made in a LA-MRSA CC398-t3015 from a broiler farm (CHAPTER 5). This isolate exhibited two different \( \text{erm}(C) \)-carrying plasmids, pSWS371 and pSWS372. The larger plasmid harbored two more genes, a \( \text{cop}-6 \) gene and a novel \( \text{pre/mob} \) gene. Besides the additional genes, both plasmids differed in their \( \text{rep} \) genes, which assigned them to different incompatibility groups and explains why they can stably coexist in the same bacterial cell.

In the second part of this Ph.D. project, the available tools to track the spread of MRSA were considered and the current knowledge about these organisms from food producing animals and food with respect to the role of MRSA to act as a food-borne pathogen were summarized (CHAPTER 7). Based on the published literature it was concluded that only the presence of MRSA in/on food intended for human consumption does not equate to MRSA being classified as a food-borne pathogen. The consumer-related aspects, e.g. age or health status, and also the adherence to kitchen hygiene play an equally important role.

In the second review (CHAPTER 8), the latest information on resistance genes so far detected in staphylococci from healthy as well as diseased livestock, wild animals and pets were described. It became obvious that more than 40 different resistance genes have been identified in staphylococci from animals, which confer resistance to virtually all classes of antimicrobial agents approved for use in animals. These resistance genes can be located on mobile genetic elements, which play a major role as carriers of antimicrobial resistance genes and facilitate the exchange of resistance genes with staphylococci of human origin but also with other Gram-positive bacteria.

In the third part of the Ph.D. project, the novel lincosamide/pleuromutilin/streptogramin A resistance gene \( \text{lsa}(E) \) was identified in an enterococcal multi-resistance gene cluster in MRSA ST398 and MSSA ST9 isolates of human origin from Spain (CHAPTER 9). The \( \text{lsa}(E) \) gene was the first \( \text{lsa} \) gene in
staphylococci which confers this resistance phenotype and confirms that there is a gene flux between \textit{E. faecalis} and \textit{S. aureus}.

The \textit{lsa(E)} gene was also identified in 16 porcine MRSA ST9-t899-IVa isolates from China (CHAPTER 10). In one of these isolates, \textit{lsa(E)} was located in a slightly different multi-resistance gene cluster on a plasmid. Consequently, the entire plasmid was sequenced to gain insight into the structure and organization of this plasmid. The multi-resistance plasmid pV7037 proved to be 40,971 bp in size and carried, besides the previously determined multi-resistance gene cluster, a functionally active \textit{tet(L)} gene, a complete \textit{cadDX} operon and also a functionally deleted variant of the \textit{β}-lactamase transposon \textit{Tn552} (CHAPTER 12). In addition, a comprehensively analysis of the two different types of multi-resistance gene clusters identified the novel spectinomycin resistance gene \textit{spw} (CHAPTER 11).

In a further study, the novel resistance genes \textit{lsa(E)} and \textit{spw} were also detected in eleven MRSA CC9 isolates from chicken (n=8) and pig carcasses (n=2) and from a butcher (n=1) from six different wet markets and a supermarket in Hong Kong, as well as in a single MRSA CC398 isolate of a nasally colonized human patient from a German hospital (CHAPTER 13). Ten overlapping PCR assays, which covered the entire cluster, and multi-resistance gene cluster-carrying contigs, which were obtained by whole genome sequencing, showed that all 12 MRSA isolates carried a large part of the pV7037-associated multi-resistance gene cluster. This part included the resistance genes \textit{aadE}, \textit{spw}, \textit{lsa(E)} and \textit{Inu(B)}. These and previous observations suggest that a enterococcal multi-resistance gene cluster with two novel resistance genes has been acquired by MRSA/MSSA CC9 and CC398 of various origins several times at different occasions in Europe and Asia.

Moreover, the novel spectinomycin resistance gene \textit{spd}, recently identified on plasmid pDJ91S, was found in 30 LA-MRSA CC398 isolates from broilers and humans on farms (n=21) or at the slaughterhouses (n=4) in The Netherlands, from turkey meat/-products (n=4) in Germany and Austria, from a diseased pig (n=1) in Germany and also in a single LA-MSSA ST433 isolate from a diseased pig in Germany (CHAPTER 14). Electrotransformation and sequencing identified the \textit{spd} gene on a novel plasmid, pSWS2889, which is 30 bp smaller and carried a different \textit{rep} gene in comparison to plasmid pDJ91S. Restriction analysis showed that 22
isolates carried plasmid pSWS2889, whereas plasmid pDJ91S was present in the remaining nine isolates.

Furthermore, the analysis of the genetic basis of elevated enrofloxacin MIC’s of ≥ 1 mg/L among 21 MRSA and 15 MSSA isolates of CC 398, CC9, CC5 or sequence type 2269 from pigs, cattle, poultry or food of poultry origin revealed different mutations in the quinolone resistance determining regions of the genes gyrA, gyrB, grlA and grlB as well as in the promoter region of norA (CHAPTER 15). In addition to the previously reported GrlA substitutions, all CC398 isolates, regardless of their origin and their methicillin resistance status, exhibited a Glu422Asp substitution in GrlB and a specific set of norA regulator mutations. The detection of four novel mutations (Ser80Leu and Glu84Asp in GrlA, Glu88Asp and Ser84Ala in GyrA) additionally illustrates the variety and the potential for mutational resistance to emerge.
Zusammenfassung

CHAPTER 19 Zusammenfassung

Sarah Wendlandt

Vergleichende molekulare Analyse von *Staphylococcus aureus* Isolaten aus intensiver Nutztierproduktion mit Schwerpunkt auf LA-MRSA von Geflügel


Im ersten Teil des Projektes wurden drei unterschiedliche Testpopulationen untersucht. Die erste Testpopulation umfasste 131 Isolate von klinisch kranken Puten und Hühnern (CHAPTER 3). Innerhalb der Putenisolate (n=80) überwog der klonale Komplex (CC) 398 und 21,2 % der Isolate erwiesen sich als LA-MRSA. Die Mehrheit der Hühnerisolate (n=51) gehörte zum CC5. Nur 9,8 % der Hühnerisolate wurden als LA-MRSA identifiziert, diese gehörten jedoch alle dem CC398 an.

In den beiden weiteren Testpopulationen wurden 37 LA-MRSA CC398 Isolate von vier verschiedenen Broiler-Mastbetriebe (Broiler n=11, Broilerstall n=15, Wohnhaus n=5, Menschen, die auf dem Betrieb wohnen/arbeiten n=6) (CHAPTER 4) und 46 LA-MRSA CC398 bzw. CC9 Isolate von Broilern auf vier verschiedenen Schlachthöfen (n=28) und den jeweiligen Schlachthofmitarbeitern (n=18) (CHAPTER 6) untersucht, um eine mögliche Übertragung zwischen den verschiedenen Reservoiren aufzeigen zu können. Die molekulare Analyse aller LA-MRSA Isolate der drei Testpopulationen wies zwei SCCmec- (IV und V), sieben spa- (überwiegend t011) und zwölf dru-Typen (überwiegend dt11a oder dt10q) nach. Die Empfindlichkeitstestung, die DNA-Mikroarray Analysen bzw. gen-spezifische PCR-Assays ergaben eine hohe Variabilität hinsichtlich der Resistenzphäno- (n=41) und -
Zusammenfassung


Im zweiten Review (CHAPTER 8) wurden die neuesten Informationen über bisher detektierte Resistenzgene bei Staphylokokken von gesunden als auch erkrankten Nutztieren, Wildtieren und Haustieren zusammengefasst. Es wurde deutlich, dass bereits mehr als 40 verschiedene Resistenzgene in Staphylokokken

Im dritten Teil des Projektes wurde das neue Resistenzgen \textit{lsa(E)}, das Resistenz gegenüber Linkosamiden, Pleuromutilinen und Streptogramin A Antibiotika vermittelt, in einem von Enterokokken stammenden Multiresistenz-Gencluster in MRSA ST398 und MSSA ST9 Isolaten von Menschen aus Spanien identifiziert (CHAPTER 9). Das \textit{lsa(E)} Gen ist das erste \textit{lsa} Gen bei Staphylokokken, welches diesen Resistenzphänotyp vermittelt. Sein Nachweis bestätigt einen Genfluss zwischen \textit{E. faecalis} und \textit{S. aureus}.


In einer weiteren Studie wurden die neuen Resistenzgene \textit{lsa(E)} und \textit{spw} sowohl in elf MRSA CC9 Isolaten von Hühner- (n=8) und Schweineschlachtkörpern (n=2) sowie von einem Metzger (n=1) auf sechs verschiedenen Frischmärkten und in einem Supermarkt in Hong Kong detektiert, sowie in einem einzeln MRSA CC398 Isolat eines nasal kolonisierten Patienten in einem deutschen Krankenhaus (CHAPTER 13). Zehn überlappende PCR-Assays, welche das gesamte Cluster abdeckten, sowie durch Gesamtgenomsequenzierung erhaltene das Multiresistenz-Gencluster tragende Contigs bestätigten, dass alle zwölf MRSA Isolate einen Großteil des pV7037-assoziierten Multiresistenz-Genclusters tragen. Dieser Teil

Des Weiteren wurde das neue Spectinomycin-Resistenzgen *spd*, welches kürzlich auf dem Plasmid pDJ91S identifiziert wurde, in 30 LA-MRSA CC398 Isolaten von Broilern und Menschen auf Bauernhöfen (n=21) bzw. auf Schlachthöfen (n=4) in den Niederlanden, von Geflügelfleisch/-produkten (n=4) in Deutschland und Österreich, von einem erkrankten Schwein (n=1) in Deutschland und auch in einem einzelnen LA-MSSA ST433 Isolat von einem erkrankten Schwein in Deutschland gefunden. Elektrotransformation und Sequenzierung identifizierten das *spd* Gen auf einem neuen Plasmid, pSWS2889, welches im Vergleich zu Plasmid pDJ91S 30 bp kleiner war und ein anderes *rep* Gen trug (CHAPTER 14). Restriktionsanalysen ergaben, dass 22 Isolate Plasmid pSWS2889 trugen, während in den restlichen neun Isolaten Plasmid pDJ91S präsent war.

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