

Transmission of MRSA along the meat supply chain

A methodological concept from farm to fork

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**von
Dipl. oec. troph Birgit Vossenkuhl**

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4 List of abbreviations

AMR	Antimicrobial resistance
AMG	Arzneimittelgesetz
BURST	Based upon related sequence types
CMRSA	Canadian MRSA
ccr	Cassette chromosome recombinase
FOX	Cefoxitin
C	Celsius
cm	Centimetre
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLI	Clindamycin
CC	Clonal complex
CPS	Coagulase- positive <i>Staphylococcus aureus</i>
CFU	Colony forming unit
CA	Community associated
CI	Confidence interval
DANN	Deoxyribonucleic acid
ECOFF	Epidemiological cut-off
ERY	Erythromycin
<i>E. coli</i>	<i>Escherichia coli</i>
et al.	Et alii
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
EU	European Union
e.g.	Example given
BfR	Federal Institute for Risk Assessment
Fig.	Figure
FUS	Fusidic acid
GEN	Gentamicin
g	Gram
HACO	Health care –associated, community-onset
HA	Healthcare associated
h	Hours
i.e.	In example
J regions	Joining regions
KANN	Kanamycin
kg	Kilogram
LZD	Linezolid
l	Litre
LA	Livestock associated

log	Logarithm
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
ml	Millilitre
MIC	Minimum inhibitory concentrations
min	Minutes
MHB	Mueller Hinton broth
MLST	Multilocus sequence typing
MUP	Mupirocin
NRL	National Reference Laboratory
no	Number
OR	Odds ratio
orfX	Open reading frame X
PVL	Panton-Valentin leukocidin
ppm	Parts per million
PBP	Penicillin binding protein
PEN	Penicillin G
PCR	Polymerase Chain Reaction
PSI	Proportional similarity index
PFGE	Pulsed- field gel electrophoresis
QS	Qualität und Sicherheit
SYN	Quinupristin/Dalfopristin
rDNA	Ribosomal DNA
RIF	Rifampicin
sec	Seconds
ST	Sequence type
SCC _{mec}	Staphylococcal Cassette Chromosome <i>mec</i>
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>spa</i>	<i>Staphylococcus aureus</i> protein A
STR	Streptomycin
SMX	Sulfamethoxazole
TET	Tetracycline
TIA	Tiamulin
TMP	Trimethoprim
US	United States
USA	United States of America
VAN	Vancomycin
VTEC	Verotoxin producing <i>Escherichia coli</i>
vs.	Versus

5 Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the most important antibiotic-resistant pathogens in hospitals and the community. Recently, a new generation of MRSA, the so called livestock associated (LA) MRSA, has emerged occupying food producing animals as a new niche. LA-MRSA can be regularly isolated from economically important livestock species including corresponding meats. The present thesis takes a methodological approach to confirm the hypothesis that LA-MRSA are transmitted along the pork, poultry and beef production chain from animals at farm to meat on consumers` table. Therefore two new concepts were developed, adapted to differing data sets.

A mathematical model of the pig slaughter process was developed which simulates the change in MRSA carcass prevalence during slaughter with special emphasis on identifying critical process steps for MRSA transmission. Based on prevalences as sole input variables the model framework is able to estimate the average value range of both the MRSA elimination and contamination rate of each of the slaughter steps. These rates are then used to set up a Monte Carlo simulation of the slaughter process chain. The model concludes that regardless of the initial extent of MRSA contamination low outcome prevalences ranging between 0.15 and 1.15 % can be achieved among carcasses at the end of slaughter. Thus, the model demonstrates that the standard procedure of pig slaughtering in principle includes process steps with the capacity to limit MRSA cross contamination. Scalding and singeing were identified as critical process steps for a significant reduction of superficial MRSA contamination.

In the course of the German national monitoring program for zoonotic agents MRSA prevalence and typing data are regularly collected covering the key steps of different food production chains. A new statistical approach has been proposed for analyzing this cross sectional set of MRSA data with regard to show potential farm to fork transmission. For this purpose, chi squared statistics was combined with the calculation of the Czekanowski similarity index to compare the distributions of strain specific characteristics between the samples from farm, carcasses after slaughter and meat at retail. The method was implemented on the turkey and veal production chains and the consistently high degrees of similarity which have been revealed between all sample pairs indicate MRSA transmission along the chain.

As the proposed methods are not specific to process chains or pathogens they offer a broad field of application and extend the spectrum of methods for bacterial transmission assessment.

6 Zusammenfassung

Methicillin-resistente *Staphylococcus aureus* (MRSA) zählen zu den bedeutendsten antibiotikaresistenten Pathogenen, die vor allem in Krankenhäusern aber auch außerhalb von Einrichtungen des Gesundheitswesens weit verbreitet sind. Seit einigen Jahren ist eine neue Generation von MRSA auf dem Vormarsch, die vor allem Nutztierbestände als neue Nische besiedelt. Diese sogenannten Nutztier-assoziierten MRSA wurden wiederholt bei wirtschaftlich bedeutenden Nutztieren sowie daraus gewonnenem Fleisch nachgewiesen.

Im Rahmen der vorliegenden Arbeit wurde ein methodischer Ansatz verfolgt, um die Hypothese einer möglichen Übertragung von Nutztier-assoziierten MRSA entlang der Lebensmittelkette vom Tier auf dessen Fleisch zu bestätigen. Angepasst an die Unterschiede in den verfügbaren Daten wurden dafür zwei neue Konzepte erstellt.

Zur Analyse der Übertragung von MRSA entlang der Schlachtkette wurde ein mathematisches Modell des Schweineschlachtprozesses entwickelt, welches dazu geeignet ist, den Verlauf der MRSA-Prävalenz entlang der Schlachtkette zu quantifizieren sowie kritische Prozessschritte für eine MRSA-Übertragung zu identifizieren. Anhand von Prävalenzdaten ist es dem Modell möglich, die durchschnittlichen MRSA-Eliminations- und Kontaminationsraten jedes einzelnen Prozessschrittes zu schätzen, die anschließend in eine Monte-Carlo-Simulation einfließen. Im Ergebnis konnte gezeigt werden, dass es generell möglich ist, die MRSA Prävalenz im Laufe des Schlachtprozesses auf ein niedriges finales Niveau zwischen 0,15 bis 1,15% zu reduzieren. Vor allem das Brühen und Abflämmen der Schlachtkörper wurden als kritische Prozesse im Hinblick auf eine MRSA-Dekontamination identifiziert.

In Deutschland werden regelmäßig MRSA-Prävalenz und Typisierungsdaten auf allen Stufen der Lebensmittelkette verschiedener Nutztiere erfasst. Um die MRSA-Daten dieser Querschnittstudie hinsichtlich einer möglichen Übertragung entlang der Kette zu analysieren, wurde ein neuer statistischer Ansatz entwickelt. Hierfür wurde eine Chi-Quadrat-Statistik mit der Berechnung des Czekanowski-Ähnlichkeitsindex kombiniert, um Unterschiede in der Verteilung stammspezifischer Eigenschaften zwischen MRSA aus dem Stall, von Karkassen nach der Schlachtung und aus Fleisch im Einzelhandel zu quantifizieren. Die Methode wurde am Beispiel der Putenfleischkette implementiert und zudem bei der Analyse der Kalbfleischkette angewendet. Die durchgehend hohen Ähnlichkeitswerte zwischen den einzelnen Proben weisen auf eine mögliche Übertragung von MRSA entlang der Lebensmittelkette hin.

Die erarbeiteten Methoden sind nicht spezifisch bezüglich Prozessketten und Pathogenen. Sie bieten somit einen großen Anwendungsbereich und erweitern das Methodenspektrum zur Bewertung bakterieller Übertragungswege.

7 Introduction

7.1 *Staphylococcus aureus*

Staphylococcus (S.) aureus is one of more than 40 species which comprise the genus *Staphylococcus*, a member of the family *Micrococcaceae* (<http://www.bacterio.net>). *S. aureus* are facultative anaerobic, gram positive cocci of about 0.7-1.2µm in diameter forming grape-like cluster. They are immobile, catalase and coagulase positive (14). *S. aureus* can persistently or intermittently colonize the skin and the mucous membranes of the upper respiratory, gastrointestinal, and lower urogenital tracts of humans and animals. Especially the anterior nares were identified as their preferred ecological niches. Approximately 37% of the general population are carriers of *S. aureus* (79). Although considered as a commensal, under appropriate conditions, opportunistic strains of *S. aureus* are enabled to cause invasive infectious diseases ranging from different forms of skin infections to life-threatening illness like pneumonia, endocarditis, bacteraemia or septicemia. Skin and mucosa injuries, the use of invasive medical devices, underlying chronic diseases or general immune suppression may predispose individuals to serious staphylococcal infections. *S. aureus* can also cause toxin-mediated diseases such as the staphylococcal scalded skin syndrome or the toxic shock syndrome (28). Nasal carriage appears to be a major risk factor for the development of infections (42). Besides its infectivity, *S. aureus* is also a leading cause of food poisoning due to the production of various enterotoxins during growth in contaminated food.

7.2 MRSA

7.2.1 Antibiotic resistance

Resistance genes within the bacterial genome encode for survival advantages over sensitive microorganisms under the presence of antibiotics. In addition to intrinsic antibiotic resistance which occurs without any additional genetic alteration, microorganisms are able to acquire resistance either by de novo mutation or horizontal gene transfer (56). In the latter process, one or more resistance genes are transported via extra-chromosomal mobile genetic elements like plasmids, integrons or transposons through transformation (transfer of free DNA), transduction (bacteriophage-mediated transfer), or conjugation (self transfer during cell to cell contact) (64). The main mechanisms of resistance are enzymatic drug inactivation, modification of the cellular target sites, reduction of drug accumulation by either decreasing the permeability of the cell membrane or increasing its export by the expression of efflux systems

and the creation of alternative metabolic pathways that bypasses the action of the antibiotic substance (69).

Soon after the introduction of penicillin into clinical practice in the 1940s, the first resistant strains of *S. aureus* have been reported (40). Penicillin resistance is mediated by the production of β -lactamase, a plasmid encoded enzyme that cleaves the β -lactam ring of the penicillin molecule, deactivating its antibacterial properties. Methicillin, a semi-synthetic penicillin which is resistant to β -lactamase, was introduced in 1959 to treat infections caused by penicillin-resistant *S. aureus* but in 1961, the first methicillin-resistant strains of *S. aureus* have emerged (38). In addition to all penicillins, MRSA isolates are also resistant to cephalosporins, carbapenems and monobactams (64).

Methicillin resistance is associated with the acquisition of the *mecA* gene which is part of the *mec* gene complex within the mobile genetic element called Staphylococcal Cassette Chromosome *mec* (SCC*mec*) (34). *MecA* codes for an alternative penicillin binding protein (PBP2' or PBP2a) located in the cell wall which has an insufficient binding affinity to all β -lactam antibiotics. Normally, β -lactams have a bactericide effect by disrupting the synthesis of the peptidoglycan layer of *S. aureus* which leads to an inhibition of the cell wall synthesis and ends in bacterial death (18). The SCC*mec* element is integrated into a specific so called integration site sequence in the staphylococcal chromosome within an open reading frame (orf) designated as *orfX* and is flanked by direct repeat sequences on both sides. So far, 11 different SCC*mec* types have been described in MRSA (7, 34, 35, 37, 47, 50, 57, 66, 82). SCC*mec* I-X harbor *mecA* whereas SCC*mec* XI carries a divergent *mecA* homologue (*mecA*_{LGA251}) which is also referred to as *mecC* (26, 33). The different types of SCC*mec* elements are characterized by the class of *mec* gene complex and the type of cassette chromosome recombinase (*ccr*) gene complex carrying a set of recombinase genes responsible for integration and excision of the cassette (32). The SCC*mec* element also contains three so called joining (J) regions. These non essential sections of the cassette have the ability to insert additional transposons or plasmids encoding further resistant determinants (36). Structural differences between the J regions within the same SCC*mec* types are used for defining subtypes (32).

Two opposing theories have been suggested to describe the molecular evolution of MRSA. While the single clone theory hypothesized that *mecA* may have been acquired just once by a common *S. aureus* ancestor (44) the multi clone theory, which is commonly confirmed, postulates that SCC*mec* was repeatedly introduced into different clonal *S. aureus* lineages (22).

7.2.2 Classification of MRSA

Healthcare associated (HA) MRSA

The classification of MRSA strains addresses both, genotypic differences as well as epidemiological and clinical characteristics of associated infection. First, the spread of MRSA was limited to hospitals and other healthcare facilities where it has become endemic and is still one of the most common multidrug resistant pathogens causing nosocomial infections worldwide. The so called HA-MRSA strains mainly carry *SCCmec* types I, II or III, are often resistant to antimicrobial classes other than β -lactams and usually lack the phage encoded genes for the virulent cytotoxin Panton-Valentin leukocidin (PVL) (13). Infections with HA-MRSA occur at least 48h after admission to hospital and are associated with increased mortality and consumption of healthcare recourses (29). Risk factors for MRSA colonization at hospital admission include recent prior hospitalization, contact to nursing homes, history of exposure to other healthcare-associated pathogens and selected comorbidities like congestive heart failure, diabetes, pulmonary disease, immunosuppression or renal failure (51).

Community associated (CA) MRSA

Since the mid 1990s distinct MRSA strains have rapidly disseminated in the common healthy population without exposure to the medical care system and related risk factors. The strains which are referred to as CA-MRSA mainly carry the smaller and more mobile *SCCmec* types IV or V are usually susceptible to non- β -lactam antibiotics and frequently carry PVL genes. Factors conducive to the dissemination of CA-MRSA include close skin to skin contact, skin cuts or abrasions, living in crowded or unsanitary conditions and share of contaminated items or surfaces (13). Infections with CA-MRSA are predominantly associated with skin and soft tissue infections but also include severe clinical syndromes like necrotizing pneumonia and sepsis (16). Clear separation of HA and CA-MRSA strains is not possible. CA-MRSA strains have also migrated into healthcare settings causing infections which would be categorized as HA-MRSA due to the history of health care exposure but in fact have onset in the community. An additional category of health care-associated, community-onset MRSA (HACO-MRSA) has been formed (41).

Livestock associated (LA) MRSA

Livestock has gained increasing significance as a zoonotic reservoir of MRSA after a distinct MRSA clone, sequence type (ST) 398, was first isolated from animals and family members of a Dutch pig farm in 2004 (76). Up to then, reports on MRSA in animals were limited to occasional detections in different companion animals or cases of dairy cow mastitis but as these strains could be assigned to typical human MRSA clones, human to animal transmission was

assumed (19, 59). In Europe and Northern America, the so called LA-MRSA can predominantly be assigned to clonal complex (CC) 398 whereas sequence Type ST9 dominates in Asian countries (45, 54, 67). LA-MRSA strains mainly carry SCC*mec* types IVa, V and a variant of type V. Besides resistance against all β -Lactams, LA-MRSA can carry several additional resistance genes against tetracycline, macrolides, lincosamides aminoglycosides, trimethoprim and fluoroquinolones (75). Recently, porcine isolates of sequence types ST398 and ST9 were reported carrying the multidrug resistance gene *cf*r on a transferable plasmid which is able to confer resistance to different antibiotic classes including the reserve antibiotic linezolid (39). Genes coding for PVL and various other virulence factors are commonly absent in LA-MRSA (4). However, PVL positive ST398 have been sporadically reported in association with human infections which demonstrates that LA-MRSA strains are quite able to acquire severe virulence factors (70, 78, 81).

7.3 MRSA and the food chain

Several investigations confirmed the presence of MRSA in different food producing animal species in Europe, North and South America and Asian countries especially in herds of pigs, but also in veal calves as well as broiler and turkey flocks (30, 46, 53, 61). In Germany, recent representative investigations revealed MRSA positive animals in 52.4% farms of fattening pigs, in 19.6% herds of veal calves and turkeys as well as in 0.7% flocks of broiler and 1.4% herds of laying hens (2, 8, 9).

Whereas the spread of HA-MRSA is clearly associated with high antibiotic consumption in healthcare settings the correlation between the emergence of LA-MRSA and a high or inappropriate antibiotic use in livestock farming is generally assumed but has not been definitely proven yet. However, as the rise of resistance in response to antimicrobial agents' exposure is inevitable the reduction of antimicrobial use in livestock farming is generally seen as the most effective approach to reduce actual resistance rates (64).

In food producing animals, antimicrobials are either used for the therapeutic or prophylactic control of bacterial infections and as growth enhancers. The therapeutical treatment of animals can be individual by oral and parenteral application, however when large groups of animals have to be treated, antibiotics are applied via feed or drinking water. At production systems with high stocking density metaphylactic therapy, which also includes clinically healthy animals, is a proven practice containing the spread of infectious diseases through large flocks. Prophylactic treatment of healthy animals can be useful to prevent the development of infectious diseases in particularly sensitive periods of livestock life, e.g.: surgery, transport, weaning or grouping of new herds (1). The use of antibiotics for growth promotion has been banned by EU legislation on animal nutrition from January 2006 due to public health risks

which are associated with the development and spread of antibiotic resistance. However, supplementing animal feed with antimicrobial agents to enhance growth is still a common practice in several countries outside the EU (55).

Since 2011, the consumption of antimicrobial agents in veterinary medicine is monitored in Germany. According to the German Drug Act (Arzneimittelgesetz AMG) and the Drug Act of the German Institute of Medical Documentation and Information pharmaceutical companies and wholesalers are obliged to report their annual sales of veterinary antimicrobials (3, 20). The total sales of antimicrobial agents in Germany decreased from 1706 t in 2011 to 1619 t in 2012. Although the use of Fluoroquinolones and 3-4 generation cephalosporins, so called last reserve antibiotics for human therapy, still play a minor role the sales of both classes has slightly increased between the years (table 1) With these results, Germany scored a medium ranking by European comparison (23).

Table 1: Sales volumes of veterinary antimicrobial agents in Germany in 2011 and 2012

The data were generated in the course of the German national reporting of sales of veterinary antimicrobial agents in 2011 and 2012 (10)

Antibiotic classes	Sales (t)		Difference
	2011	2012	
Tetracyclines	564	566	2
Penicillins	527,5	498	-29,5
Sulfonamides	185	162	-23
Macrolides	173	145	-28
Polypeptides	127	124	-3
Aminoglycosides	47	40	-7
Trimethoprim	30	26	-4
Lincosamides	17	15	-2
Pleuromutilins	14	18	4
Fluoroquinolones	8	10	2
Fenicole	6	6	0
1-2 gen. cephalosporins	2	5	3
3-4 gen. cephalosporins	3,5	4	0,5
Fusidic Acid	<1	<1	0
Nitrofurans	<1	<1	0
Nitroimidazoles	<1	<1	0
Total	1.706	1.619	87

Coming into force at April the 14th 2014, the 16th amendment of the AMG aims at a substantial reduction of the veterinary use of antimicrobials (65). Each livestock fattening farm in Germany will be legally bound to determine and register the frequency of antibiotic therapy.

In case of high antibiotic usage the farmer can be obliged to undertake measures with the intention to minimize the use of antimicrobials to an indispensable therapeutic level.

In addition to legal regulations other institutions like the German poultry association (Zentralverband der Deutschen Geflügelwirtschaft e.V.) or the Quality assurance scheme QS (Qualität und Sicherheit), started own strategies to control and reduce the veterinary consumption of antimicrobial agents in their area of responsibility (6, 52).

7.3.1 Public health relevance

Several investigations have verified that the presence of LA-MRSA on livestock constitutes a substantial health risk for farmers, slaughterhouse employees and veterinarians with frequent contact to colonized animals as well as for further household members as both animal to human and human to human transmission of LA-MRSA have been described (12, 71, 80). Direct physical contact seems to be the main transmission route. However also indirect spread of MRSA through contaminated surfaces of equipment, clothing and environmental factors, like dust or air, have been described (17, 25, 27). Although commonly less virulent than typical HA- and CA-MRSA clones, LA-MRSA of CC398 could already been linked with serious diseases such as endocarditis, pneumonia, as well as urinary tract, wound, and soft tissue infections (5, 21, 48, 62).

There is evidence for an increasing share of livestock associated strains among MRSA from humans in Germany (63). Especially in rural regions with intensive livestock farming LA-MRSA are often imported into healthcare facilities (43). The proportion of MRSA infections caused by livestock associated genetic types seems to correlate with the regional density of livestock farming (72, 74).

The detection of MRSA in livestock environments has been followed by a rising concern regarding an increased public health risk which is presumed to arise through handling or consumption of MRSA contaminated meats and products thereof. Various investigations could demonstrate a wide dissemination of LA-MRSA on foods of different animal origins including pork, veal, beef chicken or turkey (15, 24, 49, 60, 77). In general, two different hazardous situations might result from the presence of MRSA in food. As *S. aureus* is one of the leading causes of food borne intoxications MRSA strains might equally be able to produce responsible staphylococcal enterotoxins (31). In addition, contaminated food products are able to serve as a far reaching vehicle to transmit MRSA and their resistance genes into the human population. Although an association between MRSA carriage and the regular consumption of poultry has recently been shown the significance of a food based transmission route is still under discussion (73).

8 Objectives and Outline

Cross sectional investigations have shown that various species of food producing animals including pigs, cattle and poultry are frequently colonized with livestock associated MRSA strains at farm (2, 30, 58, 61). These strains can also be regularly isolated from corresponding meats (15) which is presumed to pose a risk to public health. Thereby the prevalence rates vary greatly between the different types of meat and differ from the MRSA status observed at respective primary production sectors. These results propose the hypothesis that LA-MRSA are transmitted along the meat supply chain, that slaughter and processing might play a decisive role in the MRSA prevalence levels in meats and that the extent of MRSA transmission significantly differs between the types of supply chains.

The objective of the present thesis was to develop a methodological concept for analyzing potential LA-MRSA transmission along the the meat supply chains of economically important livestock species including pigs, turkeys and cattle. To this end the following aspects have been included:

1. Literature review of MRSA in the pork production chain

The burden of MRSA in the pork production sector was analyzed by conducting a comprehensive review of the magnitude of published primary research articles in this field. Thereby, MRSA prevalence data were extracted and summarized at country level separated into the process steps primary production, slaughter and meat. The appearance of different genetic variants was compared likewise. In addition, risk factors for the within herd and between herd transmission at primary production level were summarized. A detailed analysis of the pork production process allows drawing conclusions on critical steps for MRSA growth and transmission. The public health significance of the presence of MRSA in the food chain was discussed.

2. Development of a framework for modeling MRSA transmission along the pig slaughter chain

A probabilistic model was developed to simulate the transmission of MRSA along the pig slaughter process. It is the purpose of the model to quantify the impact of the initial MRSA herd prevalence among slaughter pigs on the outcome prevalence of the carcasses, to determine potential process steps where interventions are expected to be most effective to reduce MRSA cross contamination and to evaluate the effect of various changes in the slaughter process on the outcome prevalence.

3. Selection of appropriate statistic procedures for analyzing cross sectional MRSA data sets from different stages of the food chain with the intention to draw conclusions on potential farm to consumer transmission.

MRSA transmission to poultry meat was analyzed on the example of the turkey meat production chain. As data from longitudinal investigations are lacking, a statistical approach is proposed for analyzing cross sectional MRSA data sets from different stages of the food chain in order to draw conclusions on potential farm to fork transmission. Therefore, the prevalence data and the distribution of *spa* types, *SCCmec* types and antimicrobial resistance profiles among MRSA isolated from different steps of the turkey meat production chain in Germany were compared. It is hypothesized that the degree of similarity in the distribution of the considered strain characteristics between the samples from the three process steps could allow drawing conclusions on potential MRSA transmission along the chain.

4. Tracing MRSA transmission along and between different cattle food chains

Parts of the former proposed statistical approach were included in the analysis of prevalence and strain diversity among MRSA data sets from different cattle food chains including dairy cattle, veal calves and beef animals.

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10 From pig to pork: Methicillin-resistant *Staphylococcus aureus* in the pork production chain

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From pig to pork: Methicillin-resistant *Staphylococcus aureus* in the pork production chain

Birgit Lassok*, Bernd-Alois Tenhagen

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Birgit Vossenkuhl performed all steps in preparing the review including literature search, the comparative summary and evaluation of extracted MRSA data and wrote the manuscript under supervision of Bernd-Alois Tenhagen.

10.1 Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) are a major global public health concern and might also emerge as a food safety issue. Recurrent reports have proven that pig herds are an important reservoir for MRSA, specifically of the livestock associated sequence type ST398. The high prevalence of MRSA in the pig primary production and the frequent detection of MRSA of the same types in pork and pig meat products raise the question of underlying mechanisms behind the introduction and transmission of MRSA along the pork production chain. A comprehensive review of current literature on the worldwide presence of Livestock-associated (LA)-MRSA on different steps of the pork production chain revealed that the slaughter process plays a decisive role in MRSA transmission from farm to fork. Superficial heat treatments during the slaughter process like scalding and flaming can significantly diminish the burden of MRSA on the carcasses. However, recontamination with MRSA might occur via surface treating machinery, as a result of fecal contamination at evisceration or via increased human handling during meat processing. By optimizing processes with the potential towards carcass decontamination and avoiding recontamination by effective cleaning and personal hygiene management, transmission of MRSA from pig to pork can be minimized.

10.2 Introduction

Staphylococcus (S.) aureus is known as a frequent commensal and pathogen of humans and animals. It can colonize persistently or intermittently skin and mucous membranes of the upper respiratory as well as the gastrointestinal and lower urogenital tract. Nasal carriage of the organism has been identified to be the most important risk factor for the development of infections, resulting in consequence of skin and soft tissue injury (58). Diseases, which are associated with *S. aureus*, include superficial skin infections as well as systemic infections and toxinoses (47). In livestock, *S. aureus* is particularly feared as a major cause of mastitis in dairy cows and of different types of necrosis in poultry flocks (40).

The ability of *S. aureus* to adapt to selective pressure of antimicrobials facilitated the development of resistance and induced the spread of methicillin-resistant strains in health care institutions, the community and in livestock herds. Methicillin resistance results from the acquisition of the *mecA* gene which codes for an alternative penicillin binding protein (PBP2' or PBP2a). The modified surface protein has a low binding affinity to β -Lactam antibiotics and thereby reduces their bactericidal effect. The *mecA* gene is chromosomally inserted as part of the mobile genetic element called Staphylococcal Cassette Chromosome *mec* (SCC*mec*). Depending on the type of SCC*mec*, the added DNA can also carry antibiotic resistance genes on integrated plasmids, leading to multidrug resistance (31).

Since the detection of MRSA in milk from mastitis in cows in 1972, increasing interest in animals as a reservoir for MRSA has arisen (32). Several investigations isolated MRSA from different companion and livestock animal species (62). While MRSA in companion animals are mainly associated with classical human strains, a distinct MRSA clone has emerged in livestock (21).

LA-MRSA strains are non-typable with pulsed-field gel electrophoresis (PFGE) using the standard restriction endonuclease *Sma*I. Based on multilocus sequence typing (MLST), a method of defining MRSA strains by the allelic profile of seven housekeeping genes (38), sequence type ST398 was identified to predominate in livestock (35; 56; 93). Related sequence types which share at least 5 identical sequenced housekeeping genes are grouped within the Clonal Complex CC398 using the BURST algorithm (Based Upon Related Sequence Types). The Clonal Complex is named after ST398, the ancestor strain with the largest number of single-locus variants in the group (38). Various *spa* types have been assigned to CC398, with t011, t034 and t108 being the dominating types (35). *Spa* types are defined by single locus DNA-sequencing of the polymorphic region of the *Staphylococcus* protein A gene (*spa*). The sequence and order of the repeats determine the *spa* type of the strain (41).

LA-MRSA strains mainly carry SCC*mec* types IVa, V and a variant of type V, coding for resistance against tetracycline and frequent resistance against macrolides, lincosamides aminoglycosides, trimethoprim and fluoroquinolones. The common absence of Panton-Valentine leukocidin (PVL) and various other virulence factors differentiates LA-MRSA from community-associated (CA)-MRSA strains (4). Table 2 compares the main features of LA-MRSA, HA-MRSA (hospital associated) and CA-MRSA strains.

Pig primary production was identified to be one of the most important reservoirs for LA-MRSA. Retrospective analysis of preserved isolates indicated that the clone has been present in the pig population in Germany at least since 2004 (69) which coincides with its first isolation from a pig and its farmer in the Netherlands in the same year (110).

Subsequently, the pig primary production including downstream industries was subject of numerous investigations to determine the respective LA-MRSA detection rate. The increasing number of reports of LA-MRSA in livestock-derived food products raises the question how the organism spreads at different stages of the pork production chain.

This review discusses current literature on the worldwide presence of LA-MRSA on different steps of the pork production chain with respect to prevalence and dominating lineages in different geographical regions. For this purpose, scopus <http://www.scopus.com> and <http://www.pubmed.com> where searched using the keywords “MRSA” and “*Staphylococcus aureus*” in combination with “ST398”, “CC398”, “pig, meat”, “food”, “slaughter”, “hygiene” or “hospital”. In addition, listed literature in the available studies was crosschecked.

The first part of the review compiles published investigations of LA-MRSA in the pig primary production including an overview of analyzed risk factors for the inter and intra herd transmission. The second part reviews recent findings relating to the incidence of LA-MRSA during slaughter, further meat processing and on the final meat product to draw conclusions on the critical processes for the transmission of MRSA in the pork production chain. The third part discusses the public health relevance of LA-MRSA on different steps of the pork production chain.

Table 2: Main features of the different MRSA types

	LA-MRSA	HA-MRSA	CA-MRSA
Definition	Livestock-associated MRSA: distinct strains isolated from livestock and people in close contact to livestock	Hospital-associated MRSA: strains isolated in health care settings or from patients at least 48 h after hospital admission	Community-associated MRSA: strains isolated in an outpatient setting, or from patients within 48 h of hospital admission without risk factors for HA-MRSA
PFGE	Non-typeable with standard PFGE with <i>SmaI</i> endonuclease (11)	Typeable (74)	Typeable (74)
SCC <i>mec</i>	SCC <i>mec</i> types IV and V dominating (109)	SCC <i>mec</i> types I, II and III dominating (39)	SCC <i>mec</i> types IV and V dominating (31)
MLST	Major clone: ST398 (35)	Major clones: ST8, ST250, ST239, ST247, ST5, ST228, ST22, ST36 and ST45 (39)	Major clones: ST1, ST8, ST30, ST59, ST80, ST93 (108)
Presence of PVL genes	Individual isolates (118; 119)	Rare (31)	Frequent (31)
Risk factors	Livestock: age, herd/farm size holding type and animal replacement policy, use of antimicrobials is suspected Humans: contact to colonized livestock (2; 9; 15; 25; 35; 102)	Prolonged antimicrobial therapy, prolonged hospitalization, care in an intensive care unit, surgical procedures, close proximity to a hospital patient who is infected or colonized with MRSA (109)	Gastrointestinal disease, intravenous drug use, direct contact with an individual who has a skin infection with CA-MRSA, indirect contact with contaminated objects, close contact among military recruits, travel to high-prevalence areas (31)
Resistance	Multidrug resistance (4)	Multidrug resistance (28)	Often limited to β -lactam antibiotics (31)

10.3 MRSA prevalence in the pig primary production

Since the first report about the presence of MRSA in the meat producing pig population and a regional high carriage rate among pig farmers in the Netherlands in 2005, an increasing awareness of MRSA in livestock arose (110). Several studies were conducted in various countries around the world, to assess the prevalence of MRSA, to understand the dynamics

of spread within the pig primary production sector and to appraise the public health relevance.

Within a comprehensive baseline study in 2008, the European Food Safety Authority (EFSA) detected positive breeding herds in 12 of 26 European countries. The MRSA prevalence among pig farms in the European Union was determined as 14% (0-46% range) in breeding holdings and 26.9% (0-51% range) in production holdings (35). In addition to the baseline study, various European countries carried out national or regional investigations in order to analyze the MRSA prevalence of their healthy pig herds. In Germany, investigations ascertaining the spread of MRSA in the pig primary production revealed a herd level prevalence ranging between 45 and 70% (2; 42; 59). These results were higher than the 43.5% breeding and 41.3% production farms identified by the European Union. The differences might be due to the selection of farm types. German fattening farms were consistently more often positive than breeding farms. Furthermore, the amount of positive herds seems to correlate with the pig density of the respective region. In the Netherlands, the prevalence of MRSA positive pig herds of different production types was estimated to range between 23 and 71% (15; 35; 103; 104). Particularly holdings harboring finishing pigs suffer from a high MRSA load. Between 2007 and 2008, a marked increase in the percentage of Dutch positive pig herds could be observed. The upward trend described was primarily attributed to the transmissibility of MRSA between distinct pig herds (15). From further investigations on the European continent, the presence of MRSA positive pig farms was reported from Belgium (22), Croatia (49) Denmark (63) and Portugal (83) with prevalences ranging between 16.7 and 100%. Beyond Europe, MRSA was also isolated from pigs in the primary production in Canada (56; 116), the USA (70; 93), Peru (5) and several Asian countries (3; 7; 23; 55; 61; 64; 101; 111). Table 3 summarizes available publications, including respective sample sizes and detection rates.

Comparing the molecular typing results of the MRSA isolates, regional differences in the dissemination of genetic variants can be observed. In Europe, Canada, the USA and Peru, the majority of MRSA strains from pigs in the primary production could be assigned to CC398. Sporadically occurring non-CC398 strains were assigned to CC1, CC9, CC30 and CC97. Within the CC398 lineages, t011, t034 and t108 were the most prevalent *spa* types in Europe, altogether counting for 80 and 81.3% of isolated strains from European breeding and production holdings (35). *Spa* type t108 was very common in the Netherlands, but played a minor role in the rest of Europe. In Italy, *spa* type t899 ST398 proved to be the predominating clone accounting for 27 and 24% of all isolates from pig breeding and production holdings (9; 35). Furthermore, an exceptionally high detection rate of non-CC398 strains, particularly CC1 and CC97, could be shown in the Italian pig primary production. In Canada, two human epidemic clones were identified in pig herds. Canadian (C)MRSA-2 (also known as USA100)

accounted for 14-15% of the Canadian isolates. The ST5 associated strain was reported to be the most common cause of HA-MRSA infections in humans in Canada as well as the most common strain found in colonized humans in the US. CMRSA 5 (USA500) was isolated from pigs for the first time. The strain was associated with ST8, an uncommon human epidemic strain in Canada which has been regionally reported from horses before (56; 116). In Asia, methicillin resistance seems to have emerged in a porcine *S. aureus* other than ST398. MRSA clone CC9, a minor animal MRSA sequence type in Europe and America, was predominantly isolated from swine in Thailand (5; 61), Malaysia (55; 76), China (23; 111) and Taiwan (101). The distribution of *spa* types associated with ST9 showed distinct geographic patterns, with t4358 being the most common *spa* type in pigs from Malaysia as well as t899 in China and Taiwan. A regional restricted occurrence of *spa* type t337 carrying SCC*mec* type IX was reported from pig herds in Thailand.

Comparison of these study results is limited by the use of different sampling regimes. A varying number of environmental dust samples, nasal swabs, perianal swabs or a combination of these methods was used in order to classify pig herds as MRSA positive. In addition, most reviewed investigations did not sample a statistical adequate number of pigs to make inferences about the prevalence and diversity of MRSA in the entire pig population of the country. However, investigations indicate an overall trend to a worldwide emergence of a porcine MRSA reservoir.

Table 3: Prevalence of MRSA in the pig primary production

Country	Farms			Pigs			Reference
	No. tested	No. positive	% positive	No. tested	No. positive	% positive	
Europe							
Belgium	50	34	68,0	1500	663	44,2	(22)
Croatia	8	6	75,0	68	24	35,3	(49)
Denmark	5	4	80,0	50	23	46,0	(63)
EU	1368		14,0				(35)
EU	3012		26,9				(35)
Germany	40	28	70,0	1600	169	10,6	(59)
Germany	60	27	45,0	634	211	33,3	(42)
Germany	290	152	52,4				(2)
Portugal	2	2	100,0	7	7	100,0	(83)
Portugal	12	2	16,7				(82)
the Netherlands				30	1	3,3	(110)
the Netherlands	31	7	22,6	310	35	11,3	(104)
the Netherlands	48	27	56,3				(17).
the Netherlands	50	28	56,0				(103)
the Netherlands	31	22	71,0				(15)
the Netherlands	171	115	67,3				(15)
American Continent							
Canada	20	9	45,0	285	71	24,9	(56)
Canada	46	5	10,9	460	21	4,6	(116)
Peru	6	1	16,7	120	8	6,7	(5)
USA				209	147	70,3	(93)
USA	10	5	50,0	240	7	2,9	(70)
Asia							
China	9	5	55,6				(111)
China	31	13	41,9	253	40	15,8	(23)
Malaysia	5	2	40,0	500	4	0,8	(55)
Taiwan	3	1	33,3	126	5	4,0	(101)
Thailand	4	1	25,0	40	4	10,0	(3)
Thailand	30	3	10,0				(61)

10.3.1 Risk factors for the transmission of MRSA

Among the reviewed articles, eleven studies examined the influence of a total of 26 potential risk factors on the spread of MRSA within the pig primary production. Depending on the respective study design, risk factor analysis was performed using either univariable or multivariable statistics. After multivariable analyses, pig age, herd or farm size, holding type and animal replacement policy were shown to have significant influence on the MRSA spread (2; 9; 15; 36).

Within herd prevalence

The individual MRSA colonization of pigs is subject to an inner herd dynamic. It was shown that each animal can change its MRSA status within a study period, and assumptions were made if this result might be age related. Weaning and rehousing piglets from farrowing pens to flat decks seems to increase the frequency of MRSA detection. In a longitudinal German study piglets on two independent farms were repeatedly tested for MRSA carriage during rearing. Grower/finisher pigs tended towards higher MRSA carriage rates than pigs before weaning (75). Another longitudinal investigation in a Canadian antibiotic-free farrow to feeder pig unit confirmed the German results. Over the duration of the study, the MRSA prevalence increased from 34.5% before weaning to 85% positive pigs in the post weaning period (117). Possible reasons for the increase in prevalence around the time of weaning might include an age related higher susceptibility to colonization due to specific characteristics of the commensal nasal microflora. In addition, weaning might facilitate MRSA colonization as a result of stress, confinement of negative pigs with positive fellows, or cross contamination via environmental factors or human handling (117). In contrast, an earlier Canadian study could not find a correlation between MRSA status and the age of the pigs (56). Suckling piglets also seem to be a risk group with respect to MRSA colonization. In a risk factor analysis among pigs of different age groups in the Netherlands, groups of suckling piglets reached almost identical MRSA detection rates as weanling pigs with approximately 53% positive samples, each (15). Similar results were achieved by a longitudinal investigation in a US pig production system. Younger than 12 weeks, 100% of the pigs under study carried MRSA. The prevalence declined to 50% after 18 weeks went up to 63% until week 21 and dropped to 36% in adult animals (93). It is conceivable that suckling pigs suffer from a high burden of MRSA due to age related underdevelopment of the commensal nasal microflora, which might enhance the susceptibility to colonization (117). The frequent use of antibiotics during suckling and post weaning might also contribute to the burden of MRSA in these age groups by intensifying the selective pressure (15). The MRSA prevalence of piglets was also associated with the status of the sow before farrowing. Although MRSA is not necessarily transmitted from

dam to offspring during farrowing, the probability of a piglet to become colonized was shown to be 1.4 to 2 times higher when the sow was MRSA positive (75; 114; 117). Under experimental conditions, Moodley et al demonstrated that the transmission of MRSA ST398 from positive sows to its progeny constitutes an efficient route for the vertical spread of MRSA (72).

Farm management is a decisive factor for the dissemination of MRSA, mainly influenced by herd size and production type. Intensive piggery provides optimal conditions for the introduction and transmission of MRSA. Harboring more than 500 pigs was identified as a risk factor for MRSA in finishing farms in Germany (2) and in breeding farms in the Netherlands (15). European breeding holdings with more than 400 pigs, and production holdings with more than 100 animals were twice more likely to be MRSA positive than farms harboring less than 100 pigs (36). A significant increase in prevalence was shown in Bavarian pig herds larger than 1000 animals (42) and in Italian pig herds of 9000 individuals and more (9). Herd size as a risk factor also appears to accumulate several underlying risk factors like hygiene score, purchase of gilts, the quantity of suppliers or antimicrobial use (15; 36).

Particular importance for the presence of MRSA was also attributed to different holding types. However, available investigations could not realize consistent results. The highest MRSA prevalences have been identified on finishing farms (42), wean to finish farms (2), breeding to farrowing farms (17) and on farrowing farms (59), respectively. MRSA could not be isolated from pigs in a study among German alternative farm systems (24). In contrast to conventional fattening farms, the analyzed alternative systems did not buy animal from conventional systems, raised fewer than 600 pigs, kept them on spacious lairages with straw bedding and did not administer antibiotics to animals with more than 25 kg. The isolated examination of production type as a risk factor for MRSA in pig herds is difficult as the variable might be correlated with additional factors. It could, for instance, be shown that holding types differ in the purchase frequency of pigs as well as in the number of suppliers, both potential risk factors for the introduction of MRSA into farms (2).

Although a correlation between routine use of antimicrobials and prevalence of MRSA in livestock animals was assumed repeatedly (30; 93; 104), the association has not been confirmed significantly for pig husbandry using multivariable statistics (2; 15; 17). The only significant association could be seen in a population subgroup of 16 Bavarian closed production systems. Pigs treated with up to four different antimicrobials were less colonized by MRSA compared to pigs which received more than four different antimicrobials during rearing (42).

In an in vivo experiment, a short time treatment with tetracycline significantly increased the MRSA counts in nasal samples of piglets, compared to non treated controls. However, the

extent to which the amount of MRSA in pig nasal passages is related to the spread of MRSA is not clear (72).

Between herd transmission

Animal trading and transportation is an important factor in the MRSA spread. Once introduced into the pig population, intensive trade relations accelerate the dissemination of MRSA between the herds. The amount of MRSA-positive holdings with breeding pigs is significantly associated with the number of pig imports of the country. In particular the import of pigs from countries with a high MRSA prevalence rate increases the risk of MRSA introduction (36). Farms with open production system are at risk of introducing MRSA into the stock by purchasing colonized animals. Farms with MRSA positive suppliers suffer from an 11 times higher odds for becoming positive than farms which buy pigs from MRSA free herds (17).

During transportation to the abattoir and over the waiting period in lairages before slaughter, transmission of MRSA between pigs from different farms can occur. Combining prevalence rates of 10 pig herds at farm and at slaughterhouse before stunning, a longitudinal study in the USA could show that animal transportation leads to an increase in MRSA colonization from 2.9% at farm level and 11.3% at the slaughterhouse (70). In a Dutch experimental study (16), four MRSA negative pig herds were transported to the slaughterhouse. Based on the results of nasal swabs taken on arrival at the abattoir, 10.3% of the pigs from two of the batches became MRSA positive during transport. Repeated sampling after stunning revealed an increase in MRSA prevalence up to 59.8% after the pigs had spent their resting time in the slaughterhouse lairages. Positive animals were found in all batches ranging from 6.7 to 100% in each batch. Only one truck picked up other pigs on the way to the slaughterhouse, and the animals were housed in separate sections of the truck and lairage; therefore, the MRSA must have been transmitted directly by animal contact and indirectly through cross-contamination via environmental factors. Truck drivers and abattoir personnel might also serve as vectors.

10.4 MRSA prevalence at slaughter and meat processing

When MRSA is present in the pig population, the delivery of colonized animals to the respective slaughterhouses is inevitable. As a commensal, MRSA can colonize pigs without any clinical symptoms. Without microbiological screening, it is not possible to distinguish between positive and negative herds to allow logistical slaughter.

Various investigations have been conducted to evaluate to what extent MRSA enters the slaughterhouses via colonized pigs. Samples have been taken from pigs at slaughterhouse lairages or after stunning. However, data collected at the beginning of the slaughter process cannot be used directly to infer MRSA prevalence in the primary production sector because cross-contamination during transport or in slaughterhouse lairages can raise the MRSA detection frequency in the herds.

Denmark (1), Germany (10; 54; 99), Italy (9), Switzerland (51; 80), Spain (48; 84) and the Netherlands (30) reported MRSA positive pigs with prevalences ranging between 1.3 and 64.7%. Further investigations were conducted on Tenerife (73) and on the Asian continent (7; 23; 64). The results of the available publications are summarized in table 4.

The regional distribution of *spa* types identified at stunning was similar to those observed in the primary production. Most of the porcine MRSA strains at European abattoirs belonged to *spa* types t011, t034 and t108. The latter strain was primarily identified by Dutch and Spanish investigations, accounting for 37.5 % (30) and 11% (48; 84) of all isolates, respectively. *Spa* type t899 was predominating in Italian abattoirs and could be found in 49% of the MRSA positive slaughter pig herds. In addition, 47% of the positive herds carried non-CC398 strains (CC1, CC9 and CC97) (9). Spain also investigated the MRSA prevalence among slaughter pigs on the isle of Tenerife and identified 85% positive animals (73). ST398 was exclusively isolated. Increased MRSA transmission rates in consequence of geographical characteristics of the Island were made responsible for the high MRSA prevalence. Due to the narrowness of the territory pigs have to be raised under intensive housing conditions which might facilitate the within herd spread of MRSA. Cross contamination during transportation and in lairages at the sole slaughterhouse of the island might have also contributed to an inter herd transmission.

In Asia, MRSA was reported among slaughter pigs in Korea (64), China (23) and Japan (7) with low prevalences ranging between 0.9 and 7%. Korea was the only Asian country that reported CC398 to be the dominating strain in the regional pig population. 81% of the examined slaughter pigs carried CC398 strains. The rest of the isolates could be assigned to ST72, a human associated strain. The Chinese slaughterhouse study confirmed *spa* type t899, MLST type ST9 to be the dominant sequence type in the Chinese pig population (23). One sampled pig in a Japanese abattoir was positive for MRSA t002 which was assigned to ST221 and CC5 (7).

Table 4: Prevalence of MRSA among pigs at the beginning of the slaughter process

Country	Herds			Pigs			Reference
	No. tested	No. positive	% positive	No. tested	No. positive	% positive	
Europe							
Denmark				789	101	12,8	(1)
Germany	206	177	85,9	1026	596	58,1	(99)
Germany				133	86	64,7	(10)
Germany				79	22	27,8	(54)
Italy	118	45	38,1				(9)
Spain	6	5	83,3	106	37	34,9	(48)
Spain				263	160	60,8	(84)
Switzerland				797	31	3,9	(80)
Switzerland				800	10	1,3	(51)
The Netherlands	54	44	81,5	540	209	38,7	(30)
Tenerife	15			300	257	85,7	(73)
Asia							
China	3	2	66,7	256	18	7,0	(23)
Japan	23	1	4,3	115	1	0,9	(7)
Korea	66	15	22,7	657	21	3,2	(64)

10.4.1 Transmission of MRSA along the slaughter line

Slaughter and meat processing plays an essential role in the transmission of MRSA from pig to pork. Figure 1 shows a flow diagram of the essential steps of the pork production chain.

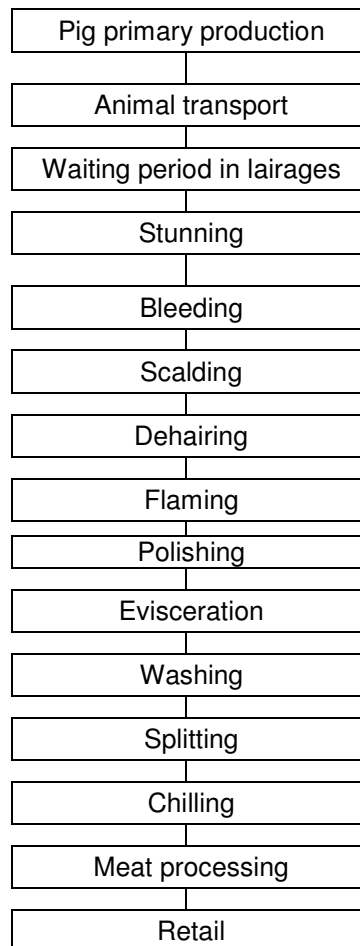


Figure 1: Process flow diagram: Pork production chain

The high entry of MRSA into the slaughterhouses via colonized pigs raises the question how and to what extent MRSA is able to spread along the process chain. Currently three studies have analyzed the prevalence of MRSA on different stages of the pork production chain (10; 54; 70). Although decisive differences in the study designs reduce the validity of result comparison, the investigations agree in a considerable reduction of MRSA along the meat processing chain. While 11.3-64.7% of the pigs carried MRSA at stunning, the prevalence decreased to 2.8-3.8% on the final products. Figure 2 compiles evaluated prevalences separately for each process step. Beneke et al. took samples on several stages of the fresh pork production process in a German abattoir and could demonstrate a successive reduction in

prevalence along the chain (10). Kastrup examined the spread of MRSA in five German abattoirs. In her thesis, higher carrier rates were found on samples at the slaughter line than at stunning. Molla et al. reported a decreasing prevalence by slaughter procedures with a subsequent increase in meat products. The observed differences in the course of prevalences of each process chain indicate that the individual hygiene performance of the corresponding abattoir may have a greater influence on the MRSA status of the final product than the carriage rate of the delivered animals. The abattoir with the highest MRSA burden at stunning had the lowest burden in their processed meat.

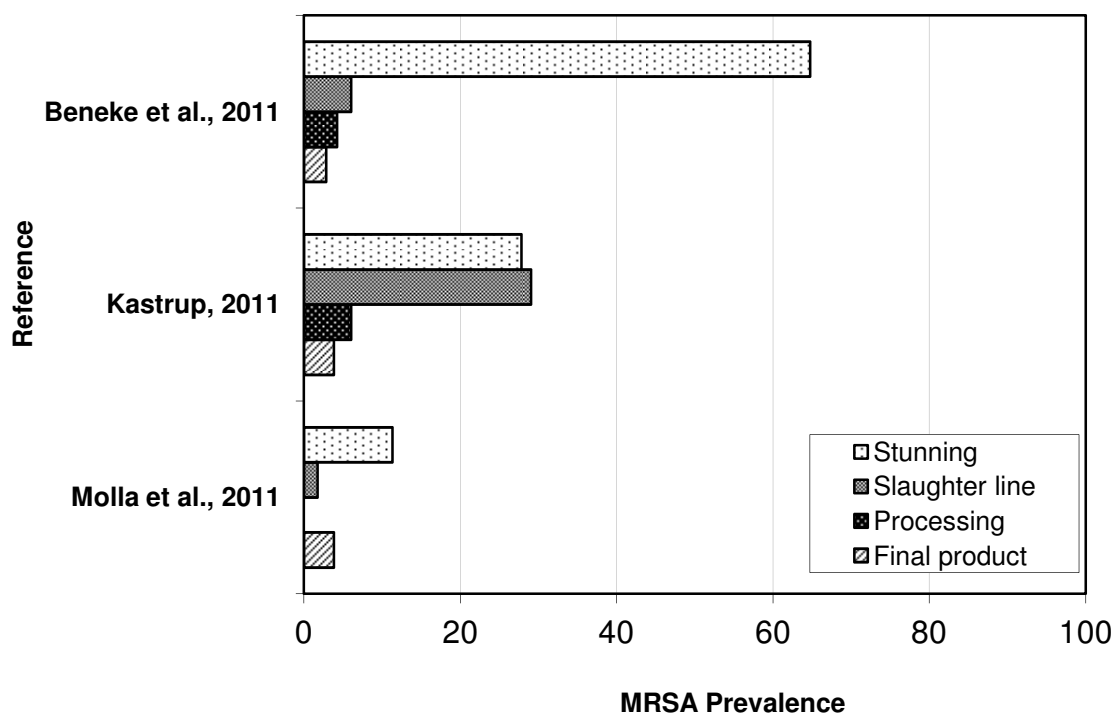


Figure 2: Prevalence of MRSA determined on different steps of the pork production chain

The numbers of studies tracing MRSA along the slaughter line is limited. Findings about the influence of single slaughter steps on microbiological carcass contamination and decontamination in general and the burden of *S. aureus* in particular could be applied to analyses of MRSA and used to draw conclusions about MRSA behavior during pig slaughter.

Several investigations that included tracing of total bacterial counts, *Enterobacteriaceae* or other indicator organisms along the slaughter line, revealed that scalding is the first slaughter process with the potential to reduce the amount of bacteria on pig carcasses. Remaining microorganism on carcasses predominantly belong to thermophilic, Gram-positive types (13; 43; 44; 77; 81; 86; 94; 100). The extent of microbial reduction depends on both the time tem-

perature conditions and the heat resistance of each microorganism. Scalding treatments usually are carried out at 60° to 62°C for 6-8 minutes (14; 95). Because *S. aureus* has a D_{55} value of approximate 66 seconds a significant reduction of MRSA during scalding can be expected (12). Gill et al. could show that staphylococci can be consistently detected in minor amounts during successive process steps (44). More recently, an analysis of the effect of certain production steps on the quantity of coagulase- positive *Staphylococcus aureus* (CPS) on pig carcasses was conducted in two Swiss abattoirs (97). After bleeding, CPS could be isolated on 96-100% of all carcasses with counts of 2.5 to 3.5 log CFU cm⁻². Scalding for 5 and 8.5 minutes at 59-62°C reduced the number of positive carcasses to 18 and 20% with counts around 1.0 log CFU cm⁻². Whereas one abattoir was able to maintain the low colonization level of CPS along the slaughter line, the proportion of positive carcasses of the second plant increased again with the final value of 99%. Recontamination could mainly be attributed to the combined dehairing-singeing operation. The fact that considerably different recontamination levels were achieved for both abattoirs emphasizes the importance of effective hygiene strategies (97).

In general, dehairing is a critical process step for cross contamination during pig slaughter (44; 45; 77; 81). The mechanical treatment of the carcass with rotating scrapers and rubber flails leads to an increased segregation of porcine bacteria from mouth, nose, skin and intestinal tract. While driving through the dehairer, the scalded carcasses can get contaminated by the detritus which accumulates in the machine. Conventional dehairing equipment is difficult to clean and in case of insufficient hygiene performance, a persisting microbiological flora can get established (86; 97). Hot water of 60-62°C, sprayed on the carcass when it is moved through the dehairer, was shown to diminish the increase of surface contamination (94; 95) Low concentrations of MRSA or *S. aureus* in scalding water as well as the infrequent detection of positive samples indicate a low impact of scalding water on cross contamination of MRSA during the slaughter process (54; 96).

Singeing has been reported to decontaminate the surface of pig carcasses significantly but published quantitative data differ widely. Using conventional automatic singeing systems with a passage of 10-15 seconds at 900°C, a reduction of total bacterial counts, ranging between 2.5 and 3 log units, is achievable (13; 14; 77; 81). In contrast, some investigations report no effect on the microflora or even an increase in surface contamination which might be due to antiquated technical facilities or hygienic deficiencies (33; 97).

Various researchers indicated that the reduction of microorganisms as a consequence of singeing is frequently reversed by polishing. Polishing systems work with scrapers and nylon brushes which are difficult to clean and therefore facilitate the accumulation of porcine microorganisms (77; 81; 94; 120). Depending on the singeing system used, certain sectors of

the carcass might be insufficiently exposed to flaming and surviving bacteria could be redistributed over the carcass during polishing (14; 44). The amount of recontamination with MRSA during polishing seems to depend on the cleaning status of the polisher as well as on the effectiveness of the singeing process.

In various investigations, an increased amount of faecal bacteria was detected on the surface of slaughter animals after evisceration and identified the intestinal tract to be the main source of contamination on this process stage (81; 86; 97; 120). As staphylococci as well as their resistant variants can regularly be isolated from the porcine intestines (98), transmission from the intestines to muscle tissue can also be expected. Post evisceration spraying with water is used to remove visible contaminants from carcasses before entering the chiller. Investigations revealed that using water of 85°C for 20 seconds can decrease existing carcass contamination whereas cold water merely distributes existing bacteria on the carcass surface (13; 46; 66).

Contrasting results regarding the influence of slaughter processes on the superficial contamination of swine carcasses with *S. aureus* were achieved on hog slaughter plants in Iowa. The percentage of positive swabs increased linearly from 4,4% after singeing and polishing to 12,6% after 24 hours of carcass chilling which was blamed on the increase in human handling with advanced slaughter (89). Investigations in a Brazilian abattoir could not find any significant influence of slaughter processes on the isolated number of *S. aureus*. Surface swabs were taken from carcasses after dehairing, before and after evisceration and splitting and after 24 hours of refrigeration. Bacterial counts between 1.2 and 1.5 log CFU/cm² could be identified on each sample moment. Although the investigated slaughter line involved decontamination steps where carcasses were sprayed with 1.5-2.0 ppm chlorine water and 0.85-3% lactic acid, no significant influence on the burden of *S. aureus* could be achieved (29).

In order to improve the microbiological status of pig carcasses at the end of the slaughter line, additional antimicrobial intervention technologies are gaining interest. The application of hot water, steam, organic acids, chlorine or different kind of salts on porcine carcasses can offer the opportunity to reduce bacterial counts by maximal 2 log (66). In accordance to article 3 (2) of the Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules on the hygiene of foodstuffs, the decontamination of meat in abattoirs of the European Union is limited to the use of drinking water (34).

To inhibit proliferation of residual MRSA on carcasses, it is important to reduce their surface temperature as rapidly as possible. Under the terms of Regulation (EC) No 853/2004, the temperature of the complete carcass has to be reduced below 7°C before further processing

if the slaughter premises do not include a separate cutting section (34). In the pork industry, pig carcasses are usually chilled over night using either conventional single stage chilling regimes or alternative cooling technologies like spray chilling, ice bank chilling in humid air at 2°C and rapid- or ultra-rapid chilling, where carcasses are initially exposed to a pre-chilling period with air at -10 to -30°C (18; 52). It is reported that *S. aureus*, originating from pigs or the environment, do not grow at temperatures below 6°C (12). Spescha et al. could show both, a 77% decreased proportion of positive carcasses and a quantitative reduction of *S. aureus* on the surface of carcasses after chilling (97). Freeze chilling (at temperatures from -10 to -25°C for 45-60 min., followed by chilling at 2°C for 23 h) was shown to reduce *Staphylococcus aureus* by 1 log CFU/cm² on untrimmed carcasses (20).

10.4.2 Meat processing

Leaving the slaughterhouse chillers, residual MRSA on carcasses' surface can be transmitted during meat processing via human hands, cutting tools and any surfaces with direct meat contact. The increase in staff employment and manual handling during processing additionally facilitates the entry of human MRSA strains into the production units. A Swiss meat processing plant reported the presence of *S. aureus* on 22.7% of the received chilled pork hind-quarters from 18 different European suppliers, harboring bacterial counts between 0.1 and 2 log CFU/cm² (92). The finding that contaminated pork could be traced back to few specific abattoirs confirms that the burden of staphylococci on pork is influenced by the slaughter process.

Besides MRSA transmission and recontamination of pork during processing, a significant reduction of the initial MRSA counts on meat loins can be expected as a result from the removal of surface tissue during the trimming procedure (89). Investigating German pork processing units, Kastrup determined a MRSA detection frequency of 6% on meat trimmings, 2% on processing equipment and 5% on employees. As the detection of MRSA positive meat trimmings was always connected with positive environmental or hand swabs, transmission of MRSA along the line is to be expected. Molecular typing of the isolates confirmed this suspicion as all MRSA from meat, plant environment and hands were identified to be *spa* type t011, exclusively (54). With 4.2% MRSA positive meat samples, Beneke et al. obtained a similar detection rate in the processing area of a German abattoir. MRSA positive environmental swabs were only detected in the slaughter area, whereas swabbed processing utensils did not contain any MRSA (10). In general, *S. aureus* is known to remain viable on stainless steel surfaces and hence might present a recontamination hazard for considerable periods of time. In an experimental setting, *S. aureus* at a contamination level of 5-7 log CFU/100 cm² dry stainless steel was detectable for at least 96 hours. Even using lower initial

concentrations of 3 log CFU/100cm², *S. aureus* could be isolated within 48 hours (60). In the Netherlands, de Jonge et al. assessed the presence of MRSA in three meat processing facilities and two institutional kitchens. MRSA could not be isolated from any human nose or hand swabs but 31 participants (33%) carried Methicillin-susceptible *Staphylococcus aureus* (MSSA). 5 meat samples (14.3%) were contaminated with MRSA. The selection of analyzed meat contained 10 pork samples 2 of which were tested t011 positive (20%) (27).

10.4.3 Final product:

If MRSA is present in the pig population of a country, it could also be recovered from corresponding final products at retail. Table 5 gives an overview of published investigations of the MRSA prevalence among pork and pig meat products.

In Europe, MRSA positive pork and pig meat products were reported from Denmark (1), Germany (10; 19; 54; 91), Spain (68) and the Netherlands (26; 105) with prevalences ranging between 1.8 and 15.8%. Combining the MRSA detection rates of different final product types, minced pig meat portions was shown to be twice as likely positive than fresh pork samples, which might be due to the processing method (19; 91). As minced meat is usually made of meat from several animals, the probability of any MRSA entry increases with the number of carcasses used, if carcass contamination rates are assumed constant. In addition, mincing meat is associated with an increase in surface area, which might improve multiplication conditions for *S. aureus*.

In accordance with the regional *spa* type distribution in the primary production and at slaughterhouses, most of the isolates could be assigned to *spa* types t011 and t034. *Spa* type t108 was only found among Dutch food samples. Positive samples from imported pork in Denmark gave evidence for the presence of MRSA in other European countries like Poland and France (1). In the USA (50; 78; 85; 112; 113; 115) and Canada (113; 115), MRSA was isolated from 3.6 to 9.6 % of pig meat products. The majority of MRSA strains in US pork products either belonged to the widespread hospital acquired sequence type USA 100 (ST5) or to USA 300 (ST8) which is the most common CA-MRSA strain in the USA. ST398 was not widespread in US meat. The reviewed investigations indicate that MRSA in the US pork chain is probably due to human contamination (50; 85; 112). No statistical difference was observed for the prevalence of MRSA when comparing between conventional and alternative pork samples originating from swine raised without antibiotics (78). Three main MRSA clones could be identified in Canada after molecular typing. Most of the MRSA were identified as Canadian epidemic CMRSA-5 and CMRSA-2, human associated strains corresponding to ST8/USA 300 and ST5/USA 100, respectively. Only a minor proportion of isolates were identified to be *spa* type t034, assigned to CC398 (113; 115). Results from a quantitative MRSA

analysis showed that 60% of the positive samples harboured 1.3 log CFU/g. The remaining contamination rates ranged between 1.5 and 3.6 log CFU/g. In contrast to the European findings, the Canadian pork chops were twice as likely MRSA positive than sampled minced meat portions (113). In Asia, MRSA on retail pork was reported from Korea and Hong Kong with prevalences of 7.1 and 21.5%, respectively (65; 79). According to results at Chinese abattoirs, Chinese pork products sampled at Hong Kong markets predominantly carried MRSA of *spa* type t899 assigned to ST9 (79). In Korea, MRSA ST72 was exclusively recovered from pork at retail (65). As mentioned earlier, ST398 was shown to dominate in the Korean pig population (64). Since ST72 is known to be the most prevalent type of CA-MRSA among humans in Korea (8), contamination during processing via staff members might be most probable.

Table 5: Prevalence of MRSA among pork and pig meat products

Country	samples			Reference
	No. tested	No. positive	% positive	
Europe				
Denmark	153	7	4,6	(1)
Germany	925	146	15,8	(19)
Germany	454	8	1,8	(91)
Germany	71	2	2,8	(10)
Germany	26	1	3,8	(54)
Spain	55	1	1,8	(68)
The Netherlands	79	2	2,5	(106)
The Netherlands	309	33	10,7	(26)
American Continent				
Canada	230	22	9,6	(113)
Canada	402	31	7,7	(115)
USA	26	1	3,8	(112)
USA	90	5	5,6	(85)
USA	55	2	3,6	(50)
USA	395	26	6,6	(78)
Asia				
Hong Kong	200	43	21,5	(79)
Korea	56	4	7,1	(65)

10.5 Public health relevance

It is considered verified by several investigations that the presence of LA-MRSA on pig farms constitutes a substantial health risk for farmers and veterinarians who come into contact with colonized animals, their excretions and contaminated dust (25; 102). Several publications show that MRSA CC398 is able to cause serious infectious diseases like endocarditis, pneumonia, urinary tract, wound and soft tissue infections (6; 37; 67; 87). The incidence of CC398 detections in hospitals as well as the proportion of MRSA infections caused by livestock associated genetic types seems to correlate with the regional density of livestock farming (105; 107). In Germany, there is evidence that the share of livestock associated MRSA among MRSA from humans is increasing (90).

The wide dissemination of LA-MRSA on pig meat products could be demonstrated by various investigations, but the public health relevance of contaminated meat remains unclear. MRSA colonization via handling or consumption of contaminated food seems to be very rare though

not impossible. So far, two clinical MRSA outbreaks have been related to the consumption of contaminated meat, but both incidences were assigned to non-CC398 strains. In the first case, a severely immunocompromised patient suffered from septicemia after ingestion of MRSA contaminated food. The causative MRSA was subsequently transmitted to several other patients via colonized nurse (57). The second incidence was a typical food intoxication caused by coleslaw, which was contaminated with toxin-producing MRSA strains (53). Investigations among professional meat handlers in the Netherlands showed that even high-frequency exposure results in a low colonization rate of not more than 3% (27). Contaminated meat could be a potential vehicle for the community spread of LA-MRSA, but following standard recommendations for hygienic handling and sufficient heating of raw meat should greatly reduce if not eliminate the risk.

The number of LA-MRSA on meat might be another reason for the restricted transmission rate. Reliable quantitative data concerning LA-MRSA on pork and pig meat products are not available, though there is some evidence that the number of MRSA on meat is low. A Canadian quantitative study among different types of retail meat identified low levels of Canadian epidemic CMRSA-2 with 37% below the detection threshold. Most quantifiable samples contained $<\log 2$ CFU/g (113). During quantitative investigations in the Netherlands, the isolation of MRSA from meat products was not possible unless sensitive pre-enrichment was used (106). Nonetheless, the possibility to develop a permanent MRSA-colonization or infectious disease after consumption or handling of MRSA contaminated meat should not be excluded, as the required infection dose has not been determined yet. Another reason for the discrepancy between the high detection frequency of MRSA CC398 and the low number of infectious diseases caused by this type of strain might be the lack of clinically important virulence factors (4; 59). Although the burden of infectious diseases caused by LA-MRSA is low so far, continuous surveillance is important as the pathogenicity potential of the clone can evolve due to insertion of additional genes. In China, five PVL-positive MRSA ST398 isolates were associated with lung and wound infections in hospitalized patients (119). The Robert Koch-Institute recently reported two PVL-positive methicillin susceptible ST398 isolated from recurrent furunculosis in Germany (88). In Italy, a worker at a dairy farm suffered from severe sepsis due to infection with MRSA ST398 and although the isolated strain did not harbor PVL-encoding genes, its virulence resembled that of PVL-positive strains.

10.6 Conclusion

Methicillin-resistant *Staphylococcus aureus* can be isolated from different consecutive steps of the pork production chain. As longitudinal interventions are rare, results of separate preva-

lence studies, which were conducted under equal regional and temporal parameters, were used to draw conclusions on the dynamics of MRSA spread along the process line. However, differences in the study design used limit the comparability of the results. In order to classify pig herds as MRSA positive, a varying number of environmental dust samples, nasal swabs, perianal swabs or a combination of these methods was used. Investigations at retail include samples of different numbers of pork and pig meat products of variable weight which were analyzed either directly following one or two enrichment steps or indirectly using swab- or rinse methods. The use of different laboratory protocols for MRSA isolation and identification, antimicrobial susceptibility testing and molecular characterization of the strains additionally hamper result comparison. Despite all differences, the reviewed investigations agree in a considerable decreasing detection frequency of MRSA from pigs at stunning to retail throughout the chain.

Pig herds are an important reservoir for MRSA. Animal age, herd or farm size, holding type and animal replacement policy were shown to have significant influence on the MRSA transmission within and between the herds. Farm level sampling in general can provide precise information about the epidemiology of MRSA in the pig primary production. However, due to small sample sizes, most of the reviewed investigations can only provide evidence of a porcine MRSA reservoir and the presence of different genetic variants in the individual countries. The national prevalence and diversity of MRSA in swine herds, however, can not be assessed on the basis of most available data sets. Sampling pigs at the abattoir before or shortly after stunning is an appropriate measure to evaluate the full extent of MRSA entry into the slaughter process. However, conclusions can not be drawn directly to the pig population prevalence, as preceding MRSA transmission during transport and in lairages can not be excluded.

With the delivery of positive pigs to the abattoirs, MRSA is able to enter the food chain. Due to the absence of specific clinical symptoms, MRSA positive animals can not be identified and separated from slaughter batches to reduce cross contamination by logistical slaughter. Nevertheless, the standard pig slaughter process seems to be able to contribute towards MRSA reduction. Especially processes including superficial heat treatment like scalding and flaming might significantly diminish the amount of MRSA on carcasses. Residual MRSA, however, can get redistributed over the carcass during dehairing and polishing via surface treating machinery. Recontamination might also occur due to faecal contamination at evisceration. The increase in manual handling during meat processing facilitates the entry of human MRSA strains into the production units. Molecular characterisation of isolated strains along the chain revealed regional differences in the distribution of different genetic clones. As identical clones are predominating both, in pigs at farm or at slaughter and on pork at retail,

MRSA on final pig meat products mostly seems to originate from animal sources and get transmitted along the chain.

Therefore it is important to analyze the slaughter process to identify critical steps for MRSA transmission. By optimizing processes with the potential towards carcass decontamination and avoiding recontamination using effective cleaning and personal hygiene management, MRSA transmission from animal to meat products can be minimized.

10.7 References

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11 Modeling the transmission of LA-MRSA along the pig slaughter line

Chapter 11 was published as

Modeling the transmission of livestock associated methicillin-resistant *Staphylococcus aureus* along the pig slaughter line

Birgit Vossenkuhl, Hannah Sharp, Jörgen Brandt, Alexandra Fetsch, Annemarie Käsbohrer, Bernd- Alois Tenhagen

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Birgit Vossenkuhl developed the model with assistance of Jörgen Brandt who coded the Markov Chain and Hannah Sharp who coded the Monte Carlo simulation. Birgit Vossenkuhl calculated all data, analyzed them in context and wrote the major part of the manuscript.

11.1 Abstract

The study introduces a new approach for a qualitative transmission assessment of MRSA throughout the pig slaughter process. Based on prevalence data found in literature the MRSA contamination and elimination rates of each individual slaughter step were estimated. The rates were used to set up a Monte Carlo simulation for modeling the propagation of MRSA along the process chain and to quantify the impact of a variable initial prevalence on the outcome prevalence of the carcasses. Sensitivity analyses for the model as well as three different scenarios were performed to estimate the impact of cross contamination during slaughter and to determine the process stages where hygiene interventions are most effective.

Regardless of the initial extent of MRSA contamination low outcome prevalences ranging between 0.15 and 1.15 % were achieved among pig carcasses indicating that the pig slaughter chain generally includes process steps with the capacity to limit carcass contamination. Especially scalding and singeing can lead to a significant reduction of superficial MRSA contamination during the first half of the slaughter process. Nevertheless, scenario analyses showed that the low MRSA outcome prevalence can only be guaranteed if recontamination during the ongoing slaughter process is obviated. In order to ensure a low MRSA load on pig carcasses at the end of slaughter the abattoir should primarily concentrate on controlling the process parameters of scalding and singeing and avoiding recontamination at subsequent process steps.

Key words: MRSA, pig slaughter chain, transmission model, Monte Carlo simulation, food safety

11.2 Introduction

Staphylococcus (S.) aureus has been relevant for the food producing industry particularly as a major cause of food born intoxications due to the production of various enterotoxins (2). As a frequent colonizer of the skin and mucous membranes, *S. aureus* can primarily enter the food chain via colonized personnel and food-producing animals (20). Standards for personal hygiene as well as cleaning and disinfection included in common recommendations for good manufacturing practice have been considered sufficient to control both the introduction and transmission of *S. aureus* during meat processing (6).

The emergence and spread of methicillin-resistant *S. aureus* (MRSA) causing severe healthcare- and community-associated infections is a major global public health concern (12, 23). The fact that *S. aureus* can rapidly adapt to the selective pressure of antimicrobials may have contributed to the wide spread observed. Beyond the well characterized burden of MRSA in healthcare and community settings, livestock has recently gained increasing significance as a zoonotic reservoir of MRSA. In Europe, these livestock associated MRSA strains (LA-MRSA) can predominantly be assigned to multilocus sequence types of clonal complex 398 (CC398)(13).

Since MRSA was first detected at a Dutch pig farm in 2004 (43), several investigations could confirm the presence of MRSA at farm level in herds of pigs (10, 13, 39) and veal calves (19, 8), as well as in broiler (7, 30, 36) and turkey flocks (8, 37).

In Germany, the prevalence of LA-MRSA was assessed at different stages of the pig production chain. Pigs at primary production were shown to be an important reservoir for LA-MRSA with prevalences ranging between 41.3 and 70% on herd level (1, 13, 15, 24). Pig prevalences between 58.5 and 80% were found among batches of slaughter pigs at the beginning of the slaughter process (42). 16% MRSA positive samples from pork and pig meat products were identified at retail in the course of a representative monitoring program throughout Germany (7) indicating transmission along the process chain. However, the relative contribution of the slaughter process to the MRSA transmission from farm to fork has not been quantified so far. Investigations could demonstrate that MRSA is present on carcasses and different slaughter equipment at various stages of the pig slaughter process (3, 21). However, MRSA prevalence data from longitudinal sampling of a sufficient number of pigs along the slaughter line are not available so far. Longitudinal investigations are cost intensive and would bring perceptible interruption of the process routine of the abattoirs under study.

In case of incomplete data, epidemiological modeling is a supplementary and cost effective method to study MRSA transmission routes in complex food production processes to estimate MRSA transmission rates and to evaluate possible control measures or intervention

strategies. In this context, two substantially different methods may be distinguished: (i) Quantitative assessment methods (28, 32) which analyze the change in the concentration of a particular microorganism along the production process and (ii) qualitative assessment methods (33) which focus on the chance of detecting a germ regardless of its concentration. Both approaches model the food production process as a modular chain of several production steps (9, 31).

The objective of this study was to describe the transmission of MRSA throughout the pig slaughter process using a qualitative model which is based on published prevalence data. The model was used to quantify the impact of the initial MRSA herd prevalence among slaughter pigs on the outcome prevalence of the carcasses, to estimate the impact of cross contamination during slaughter and to determine the process stages where interventions are most effective.

11.3 Materials and Methods

11.3.1 Data used

Assumptions concerning the transmission of MRSA from pigs to carcasses during slaughter are based on data about the presence of coagulase positive *Staphylococcus aureus* (CPS) on pig carcasses throughout the slaughter chain described by Spescha et al. (40). These data were generated in 2005 by investigations at two EU-approved abattoirs in Switzerland. Samples were obtained from the neck, belly, back and ham of 100 pig carcasses after bleeding, scalding, dehairing, singeing, polishing, trimming, washing and chilling in abattoir A and 100 pig carcasses after bleeding, scalding, a combined dehairing and singeing step, polishing, trimming, washing and chilling in abattoir B, respectively. Both abattoirs were visited weekly within 10 month and at each sampling occasion, 5 carcasses at each stage were sampled by means of the wet-dry double swab technique. All swabs were analyzed for the presence of CPS. The detection rate expressed as the percentage of CPS positive swabs out of the total number of samples was included in the model. The prevalence rates available from the two abattoirs A and B showed two different situations. In abattoir A the prevalence of CPS was reduced early in the process chain during scalding and the prevalence level was kept low throughout the remaining process steps. In abattoir B scalding also reduced the CPS prevalence to a very low level but re-contamination occurred during further processing.

11.3.2 Modeling prevalence changes throughout the pig slaughter line

A qualitative model has been developed to describe the transmission of MRSA through the pig slaughter process. Due to the process flow of abattoir B, dehairing and singeing had to be combined to a single process step in the modeled average abattoir. Therefore, the slaughter process consisted of 6 modular steps each denoted with the index i ($i = 1 \dots 6$). The state of an individual carcass at a particular production step i was denoted as S_i . An individual can have two states: positive and negative. Hence, S_i can be viewed as a random variable with two realizations: s_i^+ and s_i^- . The prevalence at a production step i , $P(s_i^+)$ can in turn be viewed as the probability of observing a positive individual at step i . If the prevalence $P(s_i^+)$ is known, the complementary prevalence $P(s_i^-)$ can be calculated as follows:

$$P(s_i^-) = 1 - P(s_i^+) \quad (1)$$

The consecutive prevalences were assumed to exhibit a first order Markov property: The individual's state at a given processing step i only depends on its state in the preceding production step $i-1$ (27). Therefore, the proposed model is completely described when all probabilities for an individual's state conditional to its state in the preceding production step $P(S_i | S_{i-1})$ are known. The quantity $P(S_i | S_{i-1})$ depends on two terms: (i) The probability of a negative individual to become positive $P(s_i^+ | s_{i-1}^-)$, which is referred to as the contamination rate and (ii) the probability of a positive individual to become negative $P(s_i^- | s_{i-1}^+)$, which is called the elimination rate. The respective complementary quantities can be calculated applying equation 1. Each individual can change its state at every processing step.

The value range of both, the contamination and elimination rate, were narrowed down by calculating their upper and lower limits from the prevalence data given by Spescha et al (40).

Based on the definition of the conditional probability of an event X given Y:

$$P(X) = P(X | Y) * P(Y) + P(X | \bar{Y}) * P(\bar{Y}) \quad (2)$$

and the definition of the respective total probability

$$P(X) = \sum_i P(X, y_i) \quad (3)$$

The following marginal distributions:

$$P(s_i^+ | s_{i-1}^+) = 1 \quad (4a)$$

$$P(s_i^- | s_{i-1}^+) = 1 \quad (4b)$$

$$P(s_i^- | s_{i-1}^-) = 1 \quad (4c)$$

$$P(s_i^+ | s_{i-1}^-) = 1 \quad (4d)$$

were used to calculate the lower and upper bounds for the contamination rate $c(\underline{c}; \bar{c})$ and elimination rate $e(\underline{e}; \bar{e})$ from the prevalences $P(s_i^+)$ and $P(s_{i-1}^+)$ in two successive production steps $i-1$ and i :

$$\underline{c} = \underline{P}(s_i^+ | s_{i-1}^-) = \begin{cases} \frac{P(s_i^+) - P(s_{i-1}^+)}{1 - P(s_{i-1}^+)} & , P(s_i^+) > P(s_{i-1}^+) \\ 0 & , else \end{cases} \quad (5a)$$

$$\bar{c} = \bar{P}(s_i^+ | s_{i-1}^-) = \begin{cases} \frac{P(s_i^+)}{1 - P(s_{i-1}^+)} & , 1 - P(s_{i-1}^+) > P(s_i^+) \\ 1 & , else \end{cases} \quad (5b)$$

$$\underline{e} = \underline{P}(s_i^- | s_{i-1}^+) = \begin{cases} 1 - \frac{P(s_i^+)}{P(s_{i-1}^+)} & , P(s_{i-1}^+) > P(s_i^+) \\ 0 & , else \end{cases} \quad (5a)$$

$$\bar{e} = \bar{P}(s_i^- | s_{i-1}^+) = \begin{cases} \frac{1 - P(s_i^+)}{P(s_{i-1}^+)} & , P(s_{i-1}^+) > 1 - P(s_i^+) \\ 1 & , else \end{cases} \quad (5b)$$

For both abattoirs A and B, values for the upper and lower bounds of the contamination and elimination rate were individually calculated for all four carcass sampling sites after each of the six processing steps. Therefore, up to a maximum of eight different values for the lower as well as upper bound of c and e for each of the process steps scalding, dehairing/singeing, polishing, trimming, washing and chilling can be achieved. All contamination rates which are based on sampling points with more than 95% positive pigs and all elimination rates based on sampling points with less than 5% positive pigs were excluded from subsequent calcula-

tions. In addition, all rates which simultaneously exhibited a lower bound of 0 and an upper bound of 1 were excluded because this means that no information on the particular rate can be obtained from these data.

For modeling the course of the MRSA prevalence along an average slaughter process, the remaining contamination and elimination rates of abattoir A and B were combined for each of the six process steps. The minimum value of all lower bounds of a process step was taken as the new lower bound (\underline{c}^* and \underline{e}^*) for the respective process step of the average abattoir and the maximum value of all upper bounds was taken as the new upper bound (\bar{c}^* and \bar{e}^*), respectively. Furthermore, the mean value of all considered rate values between the upper and lower bounds were calculated and the most likely value for the average abattoir is set as the mean of these mean values (c_{μ}^* and e_{μ}^*). The rates are then expected to follow a PERT distribution

$$c^* \sim \text{PERT}(\underline{c}^*, c_{\mu}^*, \bar{c}^*) \quad (6a)$$

$$e^* \sim \text{PERT}(\underline{e}^*, e_{\mu}^*, \bar{e}^*). \quad (6b)$$

After calculating the contamination and elimination rates of each individual process step of the average abattoir, a Monte Carlo simulation was set up for modeling the propagation of MRSA along the slaughter chain. A group of pigs enters the slaughter line with a certain fraction of MRSA positive individuals. In each process step and for each individual the probability of contamination with or elimination of MRSA is determined according to the previously calculated contamination and elimination rates for this process step. As the probability of MRSA contamination during a process step depends directly on the preceding MRSA presence, the contamination rate c^* of each process step i was multiplied with the proportion of MRSA positive individuals in the previous process step $i-1$. The model was set up by simulating 500 slaughter groups with 100 animals each.

This modeling framework allows for estimating the herd prevalence along the slaughter line for each process step and for determining the outcome prevalence dependent on a varying initial MRSA state of the herd. Sensitivity analyses were performed to determine potential process steps where interventions are expected to be most effective to reduce MRSA cross contamination. Finally, the transmission model was used to simulate various changes in the slaughter process within three different scenarios in order to evaluate resulting effects.

11.4 Results

11.4.1 Contamination and elimination rates

Table 6 summarizes the combined lower and upper bounds of the contamination and elimination rates c^* and e^* and the expected values c_{μ}^* and e_{μ}^* for each process step which were calculated for the average slaughter chain. \underline{c}^* remained 0 throughout the entire process whereas \bar{c}^* varied between 0.01 and 1. The probability for a pig to get contaminated during scalding was calculated to be 0.083. However, only one single sampling occasion could provide applicable data concerning the contamination rate of scalding. c_{μ}^* was identified to be highest during dehairing/singeing and washing with 0.45 and 0.33, respectively. However, the value range could not be narrowed down due to the high variability of the measured data originating from multiple body sites at both abattoirs. A similarly broad value range was estimated for chilling and c_{μ}^* was calculated to be low (0.09). A more precise estimation of the contamination rate was possible for polishing and trimming. With an expected value of 0.009 and 0.008, both process steps hardly contributed to contamination. \bar{e}^* was calculated to range between 0.47 and 1. For scalding, a precise estimation of the elimination rate was possible with a high expected value e_{μ}^* of 0.94. A similarly high elimination rate (0.82) could be calculated for dehairing/singeing. The value range of polishing and trimming could only be narrowed down slightly due to the variability of the underlying data. Elimination rates of 0.25 and 0.30 were estimated. A more accurate estimation could be gained for washing $e_{\mu}^* = 0.19$. The elimination rate of chilling was estimated to be 0.65.

Table 6: Calculated model parameters per slaughter process

s_i	Process steps	c^*				e^*			
		\underline{c}^*	c_{μ}^*	\overline{c}^*	n	\underline{e}^*	e_{μ}^*	\overline{e}^*	n
s_1	scalding	0	0,0833	0,1667	1	0,9000	0,9434	1	8
s_2	dehairing/singeing	0	0,4467	1	8	0,7500	0,8160	1	4
s_3	polishing	0	0,0088	0,0102	4	0,1075	0,2473	0,4699	4
s_4	trimming	0	0,0076	0,0303	4	0,0658	0,2997	0,7869	4
s_5	washing	0	0,3264	1	7	0	0,1889	0,5070	4
s_6	chilling	0	0,0877	0,8056	5	0,2674	0,6534	1	4

c^* = contamination rate

e^* = elimination rate

\underline{c}^* = lower bound of the contamination rate

\underline{e}^* = lower bound of elimination rate

c_{μ}^* = most likely value of the contamination rate

e_{μ}^* = most likely value of the elimination rate

\overline{c}^* = upper bound of the contamination rate

\overline{e}^* = upper bound of the elimination rate

n = number of observations

11.4.2 Impact of initial MRSA prevalence

The impact of the initial MRSA prevalence among the incoming slaughter pigs on the prevalence of the carcasses at the end of the slaughter process was low. As figure 3 summarizes, the variation of the initial MRSA prevalence between 5% and 95% led to a final MRSA prevalence ranging between 0.15 and 1.15 % in the basic model. Slaughter groups with a high prevalence at the beginning of the slaughter process tended to have a slightly higher contamination rate at the end of the slaughter process compared to those with a low initial prevalence.

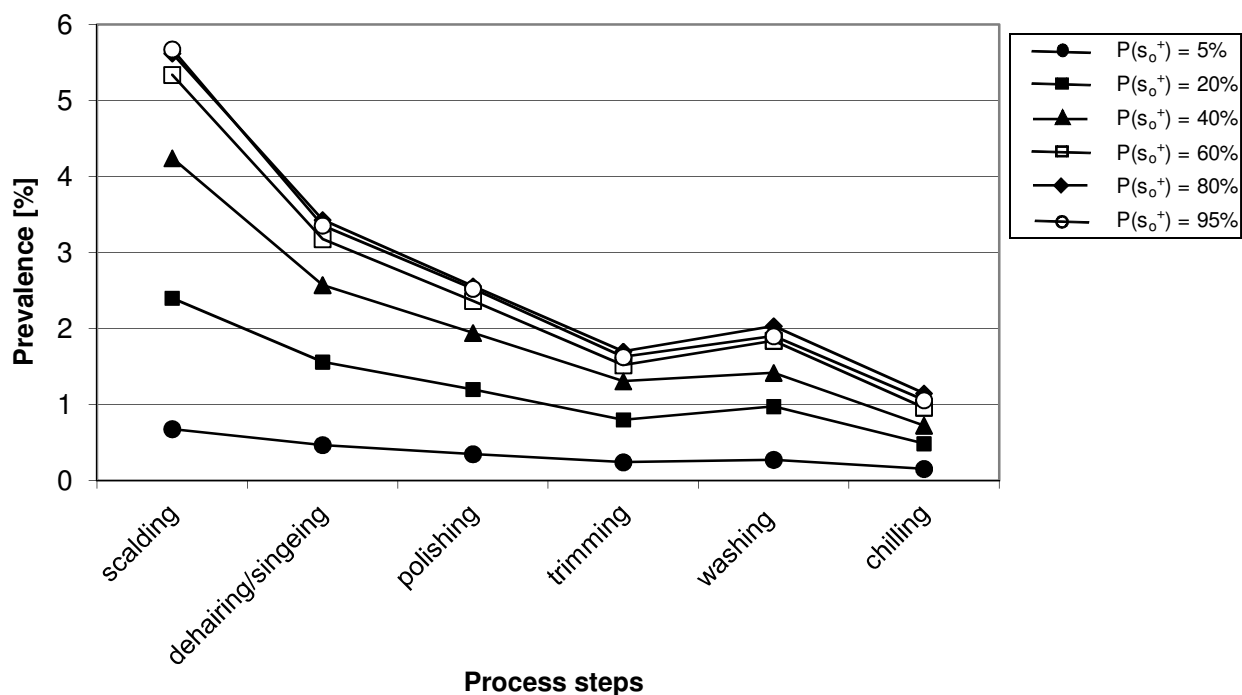


Figure 3: Change in the MRSA prevalence along the slaughter line depending on the variation of the initial MRSA prevalence $P(s_{0+})$

11.4.3 Sensitivity analysis

A sensitivity analysis was performed by changing the values of the contamination and elimination rates of every process step individually between 0 and 1 and assessing the effect on the outcome prevalence at the end of the slaughter chain. Figures 4 a/b present the final prevalences in comparison to a baseline scenario which was determined as the outcome prevalence of the simulation based on an initial prevalence of 60%, which corresponds to the MRSA prevalence among slaughter pigs in Germany (25).

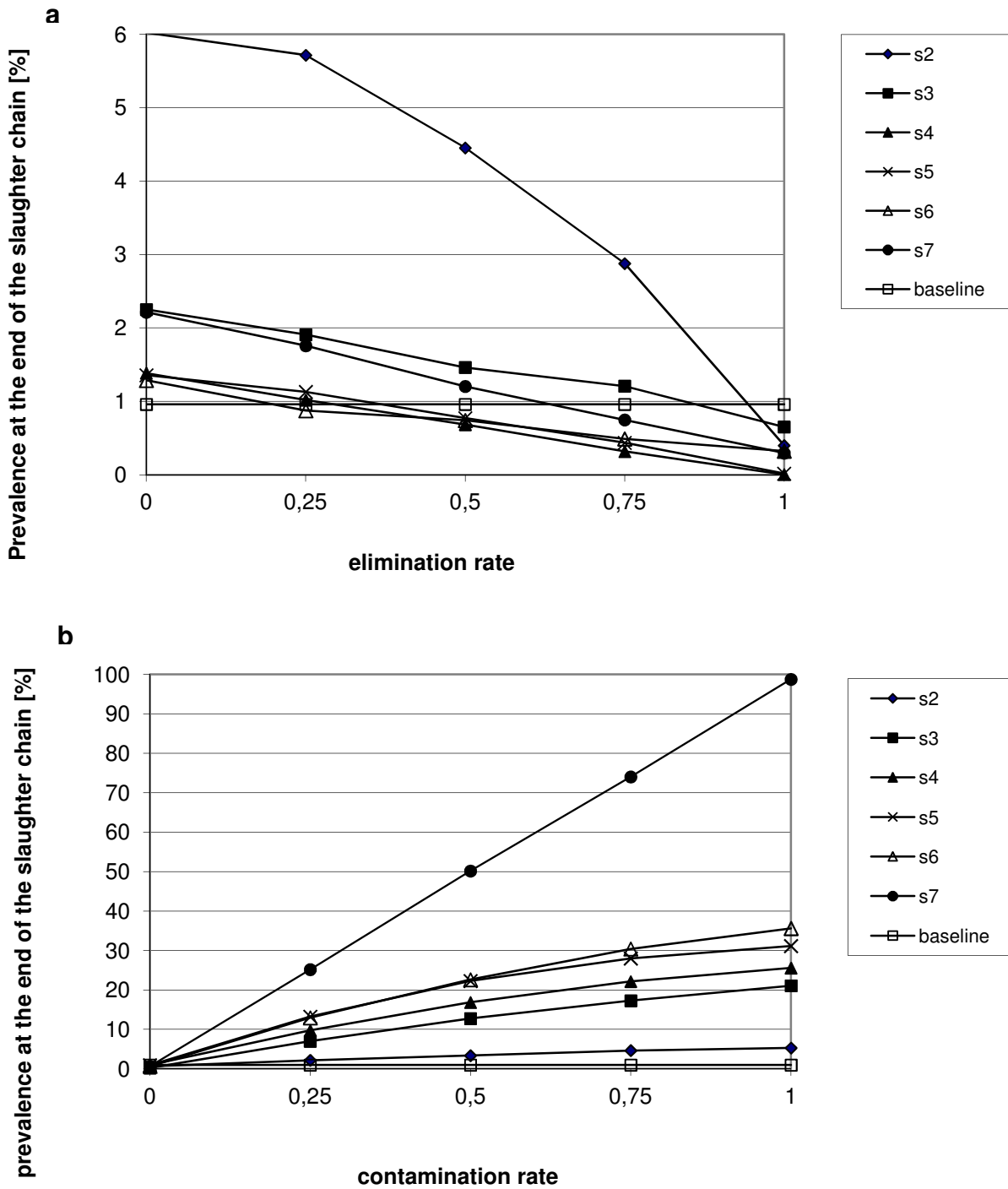


Figure 4a/b: Influence of a gradually increasing elimination and contamination rate at various process steps on the MRSA prevalence at the end of the slaughter chain

s2 = scalding, s3 = dehairing/singeing, s4 = polishing, s5 = trimming, s6 = washing, s7 = chilling

By the increase of the contamination rate in each processing step, the prevalences of the carcasses at the end of the slaughter process range between 0 and 1. The variation of the elimination rate results in final carcass prevalences between 0 and 6.02%. The increase of the contamination rate has a greater impact on the outcome prevalence than the increase of the elimination rate. The impact of changes in the contamination or elimination rate on the final prevalence is most effective if they are performed at final stages of the slaughter chain. The transmission model was also used to perform three different scenario analyses. In scenario 1, an insufficient scalding process was simulated by fixing the elimination rate to 0.5 and increasing the contamination rate by 50%. Cross contamination during dehairing/singeing and polishing was hypothesized within scenario 2. Therefore, the contamination rate of both process steps was fixed to 0.5, the elimination rates were reduced by 50%. Scenario 3 was based on scenario 2 with the addition of an increased decontamination during washing, e.g. by the use of hot water. Therefore, the elimination rate of washing was increased to 0.5 with a simultaneous decrease of the contamination rate by 50%. All scenarios were also run with an initial prevalence of 60%. All scenarios end with an increased MRSA prevalence ranging between 4.6 and 20.2% positive carcasses compared to the baseline value of 0.96%. Figure 5 summarizes the propagation of MRSA prevalences throughout the slaughter process in the three different scenarios.

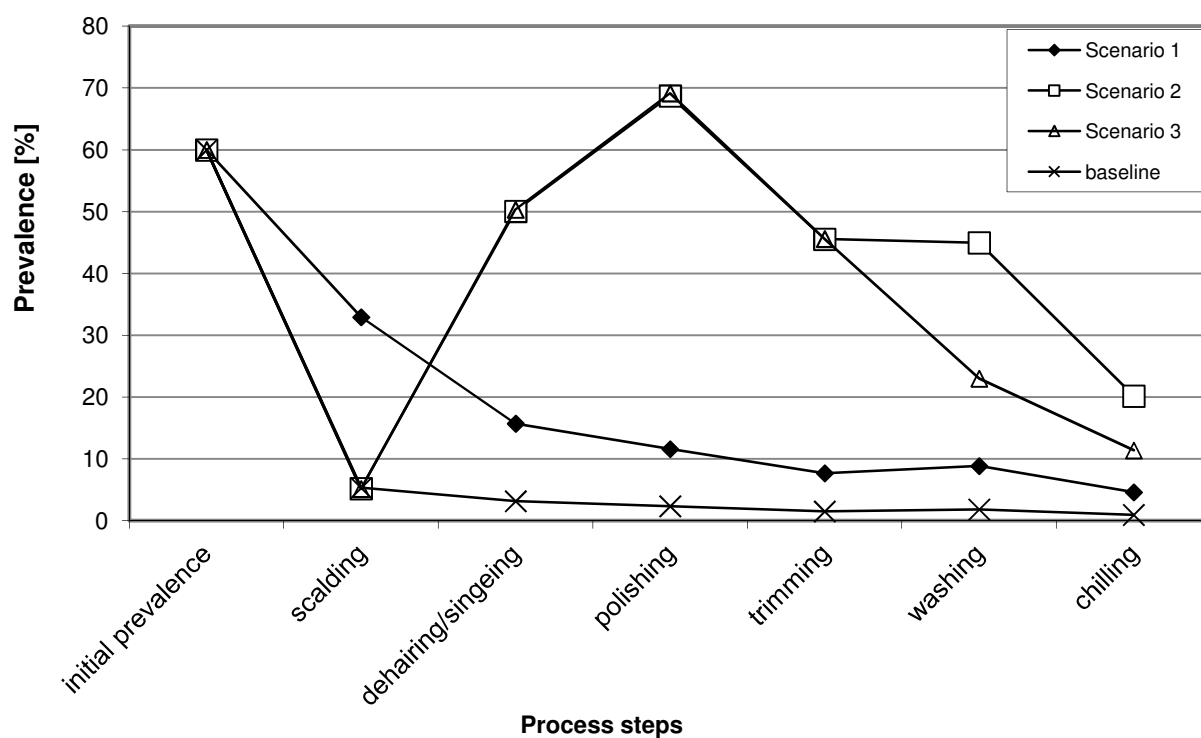


Figure 5: Course of the MRSA prevalence during three different scenarios

Initial prevalence of 60%; Scenario 1: inefficient scalding process; Scenario 2: cross- contamination during dehairing/singeing and polishing; Scenario 3: cross- contamination during dehairing/singeing and polishing, washing with hot water; Baseline scenario: course of the MRSA prevalence for the average abattoir

11.5 Discussion

The current study presents the first qualitative approach for modeling the transmission of MRSA along the pig slaughter process. The applied concept is suitable to quantify the impact of the initial slaughter batch prevalence of MRSA on the outcome prevalence of the carcasses, to identify appropriate stages for relevant hygiene interventions in the chain and to simulate the impact of cross contamination and elimination on the course of MRSA throughout the pig slaughter line. The presented model is purely based on probabilistic considerations based on prevalence data from literature. The inclusion of further assumptions based on expert opinions was avoided to achieve a model which is only based on collected data to represent the course of MRSA throughout the pig slaughter chain.

In order to model the course of MRSA along the pig slaughter process, data generated from continuous sampling of the same batch of animals both before and after each process step

are needed. Searching the literature, only an insufficient amount of investigations which have proven the presence of MRSA on pigs at different stages of the slaughter chain were available and all of the results were based on occasional sampling during the process (3, 21, 29, 42). However, one single study could be identified which investigated the prevalence of CPS on a sufficient amount of pigs at several consecutive steps along the slaughter line (40). As there is no scientific evidence of any differences between MRSA and its susceptible variant concerning the transmission and survival during the slaughter processes, the data generated from CPS by Spescha *et al.* were included in the model and applied to MRSA.

The prevalence data of CPS were used to estimate the contamination and elimination rates of MRSA for every step of the pig slaughter chain by calculating the lower and upper bounds of the rates. The exact values of the rates, however, cannot be calculated from the prevalences alone. This limitation was accepted because the presented method provides a mathematically sound way to link the separated prevalences together. When interpreting the model results, it has to be considered that the calculation of the contamination and elimination rates is based on prevalence data from only two different abattoirs. As both abattoirs show a different course of positive pigs throughout the process, a wide variability in MRSA prevalence data was observed. The degree of representativeness of the model parameter cannot be improved until unless data from a higher number of pig abattoirs are available.

Moreover, the used data were generated in 2005 and therefore, any modernization in slaughter techniques could not be considered in the model. Finally, with respect to estimating the prevalence of MRSA on carcasses, the wet-dry double swab technique probably has some limitations with respect to sensitivity (41). On the other hand, these limitations will probably only have effects on the level of the MRSA prevalence, but not on the changes in prevalence.

Assuming effective hygiene management the transmission model showed that the burden of MRSA on batches of slaughter pigs can be reduced to a low level throughout the process chain, regardless of the extent of the initial MRSA prevalence. Scalding was shown to be a particularly efficient process step for superficial carcasses decontamination. Due to the low contamination rates of subsequent process steps, the MRSA prevalence stays low until the end of slaughter.

During scalding the carcasses undergo a controlled heating process which is carried out at 60° to 62°C for 6-8 minutes (6). Since *S. aureus* is known to have a D_{55} value of approximately 66 seconds a significant reduction of MRSA during scalding can be expected (4). The elimination rate of scalding could be assessed precisely and the high most likely value of 0.94 confirms the expectations. The observed contamination rate of scalding ranges between 0 and 0.17 with a most likely value of 0.08. The calculation of this value could only be based on results generated from one carcass compartment in one abattoir. The limited diversity of

data at scalding is due to the high initial prevalence (93 to 100%) of positive pigs in the primary data source. The small number of negative animals in the sample hampers the estimation of how scalding may contribute to the contamination of pigs with MRSA. However, applying our method on data from older studies about the superficial prevalence of *Salmonella*, similar contamination rates for scalding could be observed ranging between 0 and 0.33 with a most likely value of 0.09 (data not shown) (11, 35).

Singeing is known to be another potential process step for the superficial decontamination of pig carcasses during slaughter. Conventional automatic singeing systems with a passage of 10-15 seconds at 900 to 1200°C were shown to result in a reduction of total bacterial counts, ranging between 2.5 and 3 log₁₀ CFU/cm² (5, 35). However, inefficient singeing can also lead to surviving MRSA that can be distributed over the surface of the carcasses during further processing or contaminate slaughter machines and therefore, contribute to MRSA cross contamination (11). As one abattoir in the primary data set used a combined dehairing/singeing process, separated rates for both processes could not be included into the model.

The process of trimming rather contributes to the reduction of MRSA prevalence. This result reflects the data published in Spescha *et al.* but was unexpected. Older investigations detected an increased number of faecal bacteria on the surface of slaughter pigs after evisceration, the step which directly precedes the trimming procedure in the slaughter process chain (35, 38, 44). As results from actual investigations are lacking, it can only be assumed that modernization of the slaughter technology might have also improved the hygienic status of pig carcasses after evisceration. The intestinal tract was identified to be the main source of faecal contamination on this process stage. As staphylococci including MRSA can be isolated from rectal swabs of pigs (22), transmission from the intestines to the surface of carcasses was expected. In comparison to other intestinal microorganisms like *Salmonella* or *E. coli* however, *staphylococci* play a minor role in the gut flora and therefore, recontamination with MRSA during evisceration might be low. A slight increase in the MRSA prevalence was recorded during washing. This might be, to a large extent, due to a redistribution of present bacteria on the carcass surface potentially increasing the detection rate.

Previous investigations have also shown that post evisceration washing with cold water is indeed effective in removing visible contamination but does not provide any significant reduction in the prevalence and number of bacterial counts (5, 18).

As external contamination of pig carcasses with MRSA during washing is rather unlikely, the calculated contamination rate of this process might be, to a large extent, due to a redistribution of present bacteria on the carcass surface potentially increasing the detection rate.

Sensitivity analyses showed that the variation of the contamination rate has a greater impact on the outcome prevalence than the variation of the elimination rate. This result might indicate that the pig slaughter process includes quite enough potential to reduce any superficial

MRSA contamination in the early state of the chain. The burden of MRSA on pig carcasses can be kept low by avoiding any recontamination by further slaughter steps. The impact of rate changes on the value of the final prevalence is most effective if they are performed at final stages of the slaughter chain. This effect might be partly influenced by the method used for calculating the model as due to the Markov Chain principle, the MRSA state of the individual pig at a given production step only depends on its state at the preceding production step (27). Especially cross contamination during the last part of the slaughter process can significantly increase the final prevalence of the carcasses as subsequent process steps which might dilute the contamination are lacking. As the contamination rate of each process step was multiplied with the proportion of MRSA positive individuals in the previous process step, the model concentrates on the cross contamination within the slaughter batch.

The impact of different deviances from optimal slaughter procedures was analyzed using three different scenarios. Scenario 1 simulates an ineffective scalding process which might have been realized by an insufficient water temperature, insufficient duration of scalding or cross contamination via contaminated scalding water. The resulting higher MRSA prevalence after scalding however could be reduced by subsequent process steps. Cross contamination during dehairing and polishing was simulated at scenario 2. Several previous studies concluded that dehairing is a major source of carcass contamination (11, 16, 34, 35). Rotating scrapers and rubber flails mechanically scour the surface of the carcasses to remove the bristles. The associated compression of the carcass results in an increased segregation of porcine bacteria from mouth, nose and the intestinal tract. While driving through the dehairer, the scalded carcasses can get contaminated by the detritus which accumulates in the machine (6, 17). Conventional dehairing equipment is difficult to clean and in case of insufficient hygiene performance, a persisting microbiological flora can get established (38). Various studies indicated that polishing frequently reverses the reduction of microorganisms previously achieved through singeing. Recontamination is mainly explained by the accumulation of microorganisms in the scrapers and nylon brushes of the polishing systems (35, 44). The amount of recontamination seems to depend on the cleaning status of the polisher as well as on the effectiveness of the singeing process. During singeing, certain sectors of the carcass might be insufficiently exposed to flaming and surviving bacteria might be redistributed over the carcass during polishing (6, 16). Although the high MRSA prevalence of 68.7% after polishing could be reduced during further processing, scenario 2 ends with a significantly increased proportion of positive carcasses of 20.2%.

Decontamination technologies are gaining interest in the pig slaughter process in order to reduce bacterial contamination levels or inhibit microbial growth. However, with the exception of hot water treatments, no decontamination procedures are currently authorized in the European Union (14). Scenario 3 which simulates the process of hot water spraying by increas-

ing the elimination rate of the washing process could show that this particular intervention could only induce a slight reduction of previous recontamination.

This result was in line with previous investigations which reported spraying with hot water to yield low bacterial reductions up to $3.3 \log_{10} \text{CFU/cm}^2$ (26).

11.6 Conclusion

The present study demonstrated that the transmission of MRSA throughout the pig slaughter chain can be analyzed by using a probabilistic model based on prevalence data from literature. However, data from a higher number of pig abattoirs are needed to improve the representativeness of the model parameters.

Regardless of the initial extent of MRSA contamination a low MRSA prevalence could be achieved among carcasses at the end of the chain. This finding indicates that pig slaughtering includes process steps with the capacity of superficial carcass decontamination. Especially the heat treatment during scalding and singeing can lead to a significant reduction of MRSA on the surface of pig carcasses during the first half of the slaughter process. However, scenario analyses demonstrated that low MRSA outcome prevalence can only be ensured if additionally any recontamination with MRSA is efficiently controlled throughout the ongoing slaughter process.

It can be concluded that a low burden of MRSA on slaughtered pig carcasses may be realized by a strict monitoring of important process parameters during scalding and singeing, like temperature and duration, combined with efficient hygiene practices reflected in increased elimination and reduced contamination rates of the individual pig slaughter process steps.

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12 Transmission of LA-MRSA along the turkey meat production chain

Chapter 12 was published as

Comparison of *spa* Types, SCC*mec* Types and Antimicrobial Resistance Profiles of MRSA Isolated from Turkeys at Farm, Slaughter and from Retail Meat Indicates Transmission along the Production Chain

Birgit Vossenkuhl, Jürgen Brandt, Alexandra Fetsch, Annemarie Käsbohrer, Britta Kraushaar, Katja Alt, and Bernd-Alois Tenhagen

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Birgit Vossenkuhl was responsible for the conception and implementation of all statistical analyses. She evaluated the results in context and wrote the manuscript under supervision of Bernd-Alois Tenhagen.

12.1 Abstract

The prevalence of MRSA in the turkey meat production chain in Germany was estimated within the national monitoring for zoonotic agents in 2010. In total 22/112 (19.6%) dust samples from turkey farms, 235/359 (65.5%) swabs from turkey carcasses after slaughter and 147/460 (32.0%) turkey meat samples at retail were tested positive for MRSA. The specific distributions of *spa* types, *SCCmec* types and antimicrobial resistance profiles of MRSA isolated from these three different origins were compared using chi square statistics and the proportional similarity index (Czekanowski index). No significant differences between *spa* types, *SCCmec* types and antimicrobial resistance profiles of MRSA from different steps of the German turkey meat production chain were observed using chi-square test statistics. The Czekanowski index which can obtain values between 0 (no similarity) and 1 (perfect agreement) was consistently high (0.79 – 0.86) for the distribution of *spa* types and *SCCmec* types between the different processing stages indicating high degrees of similarity. The comparison of antimicrobial resistance profiles between the different process steps revealed the lowest Czekanowski index values (0.42 – 0.56). However, the Czekanowski index values were substantially higher than the index when isolates from the turkey meat production chain were compared to isolates from wild boar meat (0.13-0.19), an example of a separated population of MRSA used as control group. This result indicates that the proposed statistical method is valid to detect existing differences in the distribution of the tested characteristics of MRSA. The degree of similarity in the distribution of *spa* types, *SCCmec* types and antimicrobial resistance profiles between MRSA isolates from different process stages of turkey meat production may reflect MRSA transmission along the chain.

Key words: MRSA - *Staphylococcus aureus* – turkey meat production chain - antimicrobial resistance

12.2 Introduction

Staphylococcus (S.) aureus is a common cause of food poisoning due to the production of various enterotoxins. *S. aureus* is a frequent colonizer of the skin and mucous membranes and therefore, personnel and food-producing animals are the main sources of *S. aureus* in food (28). The control of *S. aureus* is routinely considered in the food producing industry if standard food safety management systems are operated. In recent years, methicillin-resistant *Staphylococcus aureus* (MRSA), previously known as a multidrug resistant pathogen causing severe healthcare associated and community acquired infections, (35) has been observed worldwide in livestock husbandry as well as in food of different animal origins raising concerns about a possible farm to fork transmission.

First reported from pigs in the Netherlands (64) and France (4) a distinct MRSA lineage, Clonal Complex (CC) 398, has emerged in food producing animals in Europe especially in herds of pigs (13, 20, 33, 57), veal calves (25) broiler flocks (45, 47) and turkeys (53). Therefore, the term “livestock-associated MRSA” (LA-MRSA) was introduced considering livestock to form a new and separate reservoir for MRSA (51). In Asian countries, however, sequence type ST9, a separate genetic lineage, is predominating among MRSA isolates from livestock animals (2, 65). Different DNA sequencing methods are used for typing MRSA strains. In order to define MRSA clones, Multilocus sequence typing (MLST), a method of classifying MRSA strains by the allelic profile of seven housekeeping genes, is used in conjunction with PCR analysis of the staphylococcal chromosomal cassette *mec* (*SCCmec*), a mobile genetic element that contains the *mec A* gene encoding for resistance to methicillin (12). 11 different *SCCmec* types have been described, so far. The class of *mec* gene complex and the type of *ccr* gene complex carrying a set of recombinase genes responsible for integration and excision of the cassette characterize the different types of *SCCmec* elements (30). Whereas *SCCmec* I-X harbor *mecA* *SCCmec* XI carries a divergent *mecA* homologue (*mecA*_{LGA251}) (24). *Spa* typing differentiates MRSA strains by the number of tandem repeats and the sequence variation in region X of the protein A gene (*spa*) and can be used for reliable and discriminatory typing of MRSA (23). As particular MLST have shown to be associated with specific repeats and repeat successions it is, with few exceptions, possible to infer an MLST type from the *spa* type. (<http://www.spaserver.ridom.de>). The frequent use of antimicrobials in animal production is suspected to facilitate the emergence and spread of MRSA due to antimicrobial selection pressure (16, 48, 68). High stocking density in intensive food animal production holdings and intensive animal trading promote the rapid spread of MRSA between livestock populations (1, 8). LA-MRSA strains have also been detected in raw meat at retail including beef, veal, pork and poultry (15, 27, 39, 41, 46, 50, 62, 66, 67) indicating potential

transmission along the chain due to cross contamination during slaughter and processing. However, the extent of this transmission is so far poorly understood.

In Germany, the national monitoring for zoonotic agents aims at characterizing the prevalence of potential zoonotic pathogens at different stages of various food chains. The monitoring is part of the official control of foodstuffs and fulfills the requirements of EU Directive 2003/99/EC (18). In 2010, the turkey meat production chain was addressed in this monitoring scheme.

The objective of the present study was to use data from the national monitoring of zoonotic agents in the food chain to obtain a comprehensive insight into the presence and transmission of MRSA in the German turkey meat production chain. A new approach is proposed for analyzing a cross sectional MRSA data set from different stages of the food chain in order to draw conclusions on potential farm to fork transmission. For this purpose, the prevalence of MRSA and the distribution of *spa* types, *SCCmec* types and antimicrobial resistance profiles among MRSA isolated from different steps of the turkey meat production chain were compared. It is proposed that the degree of similarity in the distribution of *spa* types, *SCCmec* types and antimicrobial resistance profiles between the samples from the three process steps may be interpreted as reflecting MRSA transmission along the chain.

12.3 Materials and methods

12.3.1 Study design

Sampling was conducted in 2010 by the competent authorities of the federal states according to a pre-defined protocol in the framework of the national monitoring for zoonotic agents. All participating competent authorities are listed in table 7. Dust samples from 112 German turkey flocks were collected in order to quantify the presence of MRSA in primary production and to assess the introduction of MRSA into the slaughterhouses. Samples at slaughterhouses (n=359) were analyzed to estimate the transfer to carcasses during slaughter and to determine the transmission of MRSA from carcasses to fresh turkey meat during further processing. Finally, 460 turkey meat portions were sampled to evaluate the MRSA exposure of consumers via contaminated turkey meat.

Turkey pens were sampled by pooling 5 dust swab samples, collected from different sections representing an area of 500cm², each. At the slaughterhouse, at least 30 g neck skin was sampled from turkey carcasses after slaughter and chilling, but prior to further processing. Samples of 25 g of fresh turkey meat (with or without skin) were collected at retail. In order to ensure a high level of representativity, the distribution of the samples in primary production and at slaughter across Germany was proportional to the number of turkey flocks and the

slaughter capacity of the respective federal state. Meat samples at retail were distributed according to the human population size of the executive federal state. A more detailed description of the principles of the national monitoring for zoonotic agents has been published before (32).

Table 7: List of competent authorities of the German federal states

The authorities were responsible for collecting the samples

Federal States	Agencies
Baden-Württemberg	Chemical and Veterinary Investigatory Office of Stuttgart
	Chemical and Veterinary Investigatory Office of Karlsruhe
	Chemical and Veterinary Investigatory Office of Freiburg
	Chemical and Veterinary Investigatory Office of Sigmaringen
Bavaria	Bavarian State Office for Health and Food Safety
Berlin/Brandenburg	State Laboratory Berlin Brandenburg
Bremen	State Investigatory Office for Chemistry, Hygiene and Veterinary Medicine
	State Institute for Food Safety, Health Protection and Environmental Investigations
Hamburg	
Hesse	State Laboratory Hesse
Mecklenburg-Vorpommern	State Office for Agriculture, Food Safety and Fisheries
Lower Saxony	State Office for Consumer Protection and Food Safety
North Rhine-Westphalia	State Veterinary Investigatory Office Arnsberg
	Chemical and Veterinary Investigatory Office Münsterland – Emscher – Lippe
	Chemical and Veterinary Investigatory Office East-Westphalia- Lippe
	Chemical and Veterinary Investigatory Office Rhine-Ruhr-Wupper
Rhineland-Palatinate	State Investigatory Office Koblenz
Saarland	State Office for Consumer Protection
Saxony	State Investigatory Institute for Health and Veterinary Service Saxony
Saxony-Anhalt	State Office for Consumer Protection Halle
Schleswig Holstein	State Laboratory Schleswig Holstein
Thuringia	Thuringia State Office for Consumer Protection

12.3.2 MRSA isolation

MRSA were isolated by the regional laboratories according to the recommended method of the National Reference Laboratory (NRL) for staphylococci including *S. aureus* at the Federal Institute for Risk Assessment (BfR). The dust samples were pooled per turkey house in 100ml Mueller Hinton broth supplemented with 6.5% NaCl for pre-enrichment. Neck skin samples (at least 30g), fresh meat (25g) and meat preparations (25g) were pre-enriched in 225 ml Mueller Hinton broth supplemented with 6.5% NaCl. After incubation for 16-20 h at 37°C, 1 ml pre-enrichment broth was transferred into 9 ml of tryptic soy broth supplemented with 50 mg/l aztreonam and 3.5 mg/l ceftiofur. After incubation of this selective-enrichment broth for a further 16-20 h at 37°C one loopful was plated onto sheep blood agar and chromogenic MRSA screening agar respectively, and incubated for 24-48 h at 37°C. Presumptive MRSA isolates were sent to the NRL for MRSA confirmation and characterization. The number of MRSA isolates included in further analyses is not exactly congruent to the amount of positive samples obtained within the national monitoring for zoonotic agents because first, the NRL did not always receive the corresponding isolate from the competent authorities of the federal states or second, isolates which did not exactly correspond to the monitoring sampling plan in terms of completeness of data reporting to the national level but were obtained from the correct matrix were excluded from prevalence estimations but included in further typing and strain comparisons.

Twenty one MRSA isolates from wild boar meat within the national monitoring for zoonotic agents of 2011 were used in the analyses as a control group (data not shown in detail). The control group was selected to ensure wide differences with the population under study concerning the distribution of MRSA strains in order to evaluate if the used analytical approach is appropriate to differentiate between the matrices.

12.3.3 Molecular typing

Presumptive MRSA isolates were confirmed by an in-house multiplex PCR simultaneously targeting the 23S rDNA specific for *Staphylococcus* species (60), the nuclease gene *nuc* which is specific for *S. aureus*, and the resistance gene *mecA* (49). Template DNA was extracted using the "RTP Bacteria DNA Mini Kit" (Invitex, Berlin, Germany). All MRSA isolates were further characterized using *spa* typing (56) and *SCCmec* typing (69). The method applied for typing of the *SCCmec* differentiates *SCCmec* types I to V and their subtypes. However, isolates of the CC398 characterized as type III by the method have been shown to rather be a variant of type V (31). The software Ridom Staphytype (Ridom GmbH, Würzburg, Germany) was used to assign *spa* types. *Spa* types which have not been identified and as-

signed to a clonal complex (CC) by the NRL before were additionally subjected to multilocus sequence typing (MLST) (21).

12.3.4 Antimicrobial susceptibility testing

All isolates were tested for the susceptibility to antimicrobials using broth microdilution in accordance with Clinical and Laboratory Standards Institute guidelines (11). Commercial microtitre plates were used (TREK Diagnostic Systems, Magellan Biosciences, West Sussex, England). Minimum inhibitory concentrations (MIC) were evaluated according to epidemiological cut-off values (ECOFFs) published for MRSA and *S. aureus* by the European committee for antimicrobial susceptibility testing (www.eucast.org). MIC values above the ECOFFs indicated microbiological resistance. MIC lower or equal to the ECOFFs characterised susceptible strains. *S. aureus* strain ATCC 25923 was used for quality assurance. Resistance testing included gentamicin, kanamycin, streptomycin, chloramphenicol, ciprofloxacin, tetracycline, clindamycin, erythromycin, mupirocin, linezolid, vancomycin, quinupristin/dalfopristin, penicillin, fusidic acid, ceftiofur, trimethoprim, sulfamethoxazole, rifampicin and tiamulin.

12.3.5 Statistical analysis

The chi square test of homogeneity was used to analyze differences in the distribution of *spa* types and antibiotic resistance profiles between MRSA strains from the turkey flocks, carcasses at slaughter and meat. Isolates were grouped according to their *spa* types and antibiotic resistance profiles to assure appropriate numbers of isolates in all categories. All *spa* types were aggregated in accordance to their frequency of occurrence. The phenotypic antimicrobial resistance profiles were grouped by hierarchical cluster analysis using Ward's minimum variance and squared Euclidean distance. The MIC values for each isolate were categorized into resistant or susceptible according to the ECOFFs to generate a binary data set. The final amount of clusters was determined using the Pseudo-F (10) and Pseudo-T (17) statistics. Both tests indicate possible breakpoints for splitting the data into the appropriate amount of clusters. The distribution of SCCmec types in the different matrices were compared using Fisher's exact test as 33.3% of the cells of the contingency table had an expected value below 5. P-values of <0.05 were considered statistically significant. Chi square test, Fisher's exact test and cluster analysis were calculated using the statistical software package SPSS 18.0 (SPSS Inc. Munich, Germany). Pseudo-F and Pseudo-T statistics were performed using SAS/STAT software 9.2 (SAS Institute Inc., Cary, NC, USA).

The degree of similarity between the frequency distributions of *spa* types, SCCmec types and resistance profiles of MRSA among the sample sets from the turkey primary production, carcasses at slaughterhouse and turkey meat at retail was estimated using the Czekanowski index or proportional similarity index (PSI) (54). It is calculated by:

$$PS = 1 - 0,5 \sum_i |p_i - q_i| = \sum_i \min(p_i, q_i)$$

where p_i and q_i represent the proportion of strains out of all strains among the data sets P and Q which agree in the realization i of the variable of interest. The values for PS range from 1 for identical frequency distributions of the variable of interest to zero for no similarities between the data sets. Since the size of the samples is rather small, a realization of the PSI index may deviate largely from its true value. Thus, the PSI was bootstrapped obtaining a probability density distribution from which we derived the 95% confidence interval for the PSI. The statistic open source software R (available at: <http://www.R-project.org>) was used to calculate the approximate confidence interval of the Czekanowski index using the bootstrap method utilizing 1000 iterations (19).

12.4 Results

Twenty two (19.6%) of 112 dust samples from the turkey primary production, 235 (65.5%) of 359 turkey carcasses after slaughter and 147 (32.0%) of 460 turkey meat samples at retail were tested positive for MRSA (9). A set of 32 isolates from dust samples, 248 isolates from turkey carcasses and 241 isolates from turkey meat was used for further laboratory analyses (table 8).

A total of 16 different *spa* types were identified. The number of different *spa* types increased during processing from 5 different types in dust samples over 8 in carcasses to 15 different types in meat samples. The proportion of strains assigned to CC398 ranged between 85.9 and 90.6%. Among CC398, t011 (43.8-46.9%) and t034 (32.0-43.8%) were the predominant *spa* types on every process step. *Spa* types t1430 (4.0-6.3%) and t002 (3.1-9.1%) were dominating within the group of non CC398 strains.

Most of the strains carried *SCCmec* type V (58.1-71.9%) followed by type IVa (19-27.0%). Type III (0-1.2%) was identified sporadically (table 8). However, there is evidence in former literature that CC398 strains which were identified as *SCCmec* type III by the typing scheme of Zhang et al. (69) are rather assigned to a separate variant of *SCCmec* type V (3, 31, 36). In 5.7-17.6% of the strains the *SCCmec* type could not be identified by the method used.

Table 8: MRSA prevalence and distribution of spa types, SCC mec types and antimicrobial resistance clusters

The isolates were sampled at different steps of the German turkey meat production chain in 2010.

Process step	Primary production		Slaughter		Meat		total	
Samples (n)	112		359		460		931	
MRSA positive samples (n)	22		235		147		404	
MRSA prevalence (%)	19,6		65,5		32			
No. of isolates included in further statistics ^a	32		248		241		521	
Genetic Typing								
CC398	<i>spa</i> types	n	%	n	%	n	%	n
	t011	14	43.8	113	45.6	113	46.9	240
	t034	14	43.8	105	42.3	77	32.0	196
	t108	-		-		1	0.4	1
	t571	1	3.1	-		1	0.4	2
	t899	-		1	0.4	5	2.1	6
	t1255	-		-		3	1.2	3
	t1344	-		-		3	1.2	3
	t1580	-		-		1	0.4	1
	t2510	-		-		1	0.4	1
	t2576	-		1	0.4	-		1
	t2970	-		3	1.2	1	0.4	4
	t4652	-		1	0.4	1	0.4	2
	total		29 90.6	224	90.3	207	85.9	460
non CC398	<i>spa</i> types							
assigned MLST types								
ST5	t002		1 3.1	14	5.6	22	9.1	37
ST5	t010	-		-		1	0.4	1
ST45	t015	-		-		1	0.4	1
ST9	t1430	2	6.3	10	4.0	10	4.1	22
	total	3	9.4	24	9.7	34	14.1	61
total		32	100	248	100	241	100	521

		SCC <i>mec</i> Types						
	n.t. ^b	2	6.3	24	9.7	33	13.7	59
	<i>mec</i> III	-		1	0.4	3	1.2	4
	<i>mec</i> IV _a	7	21.9	47	19.0	65	27.0	119
	<i>mec</i> V	23	71.9	176	71.0	140	58.1	339
total		32	100	248	100	241	100	521
		Resistance profiles ^c						
	Cluster A	17	53.1	121	48.8	97	40.2	235
	Cluster B	10	31.3	82	33.1	88	36.5	180
	Cluster C	5	15.6	45	18.1	56	23.2	106
total		32	100	248	100	241	100	521

^a MRSA isolates which did not exactly correspond to the monitoring sampling plan in terms of completeness of data reporting to the national level were excluded from prevalence estimations but included in further typing and strain comparisons.

^bNot typable

^cResistance cluster were calculated using Ward`s minimum variance with squared Euclidean distance

Susceptibility to 19 different antimicrobial agents was determined (figure 6). Throughout the turkey production chain, the vast majority of isolates was resistant to tetracycline (98.8%-100%). High resistance rates were obtained to clindamycin (79.4-93.8%), erythromycin (73.8-87.5%), trimethoprim (65.7-78.1%), quinupristin/dalfopristin (62.2-66.1%) and tiamulin (52.3-65.6%). Resistances to mupirocin, linezolid, sulfamethoxazole and rifampicin were observed sporadically in individual isolates from all steps of the process chain. All isolates were susceptible to vancomycin. Resistance to tiamulin (62.2 versus 8.2%), gentamicin (25.2 versus 6.6%) and trimethoprim (72.0 versus 36.1%) was considerably more frequent among CC398 than among non-CC398 strains. Resistance to ciprofloxacin was common among non-CC398 strains (98.4 versus 26.1% in CC398 strains).

All 521 MRSA strains were included in further similarity estimations. In accordance to the frequency of their occurrence all *spa* types were aggregated in 4 different categories for further statistical analysis. The most prevalent *spa* types t011 and t034 built their own group whereas rare *spa* types of CC398 and all non CC398 strains were summarized in separate groups. The chi square distribution of the *spa* type groups did not significantly differ between primary production, carcasses at slaughter and meat at retail ($p=0.06$). Likewise, no significant difference was identified in the distribution of SCC*mec* types between the origins using fisher's exact test ($p=0.095$). A total of 101 different resistance profiles were identified among the MRSA isolates including resistance to 2 to 12 different antimicrobial substances. The hierarchical cluster algorithm of Wards minimum variance combined with squared Euclidean distance separated the antimicrobial resistance profiles into homogenous clusters. Identical resistance phenotypes did not appear in more than one cluster. Based on the Pseudo-F and Pseudo-T statistics the 3 cluster solution containing 33, 44 and 24 different phenotypic resistance profiles, respectively, was identified to best describe the binary data set. Detailed characteristics of the cluster composition, concerning antimicrobial resistance and the distribution of groups of *spa* types and SCC*mec* types, is summarized in table 9. The antimicrobial resistance clusters did not significantly differ in their chi square distribution between the MRSA samples from the three origins ($p=0.295$).

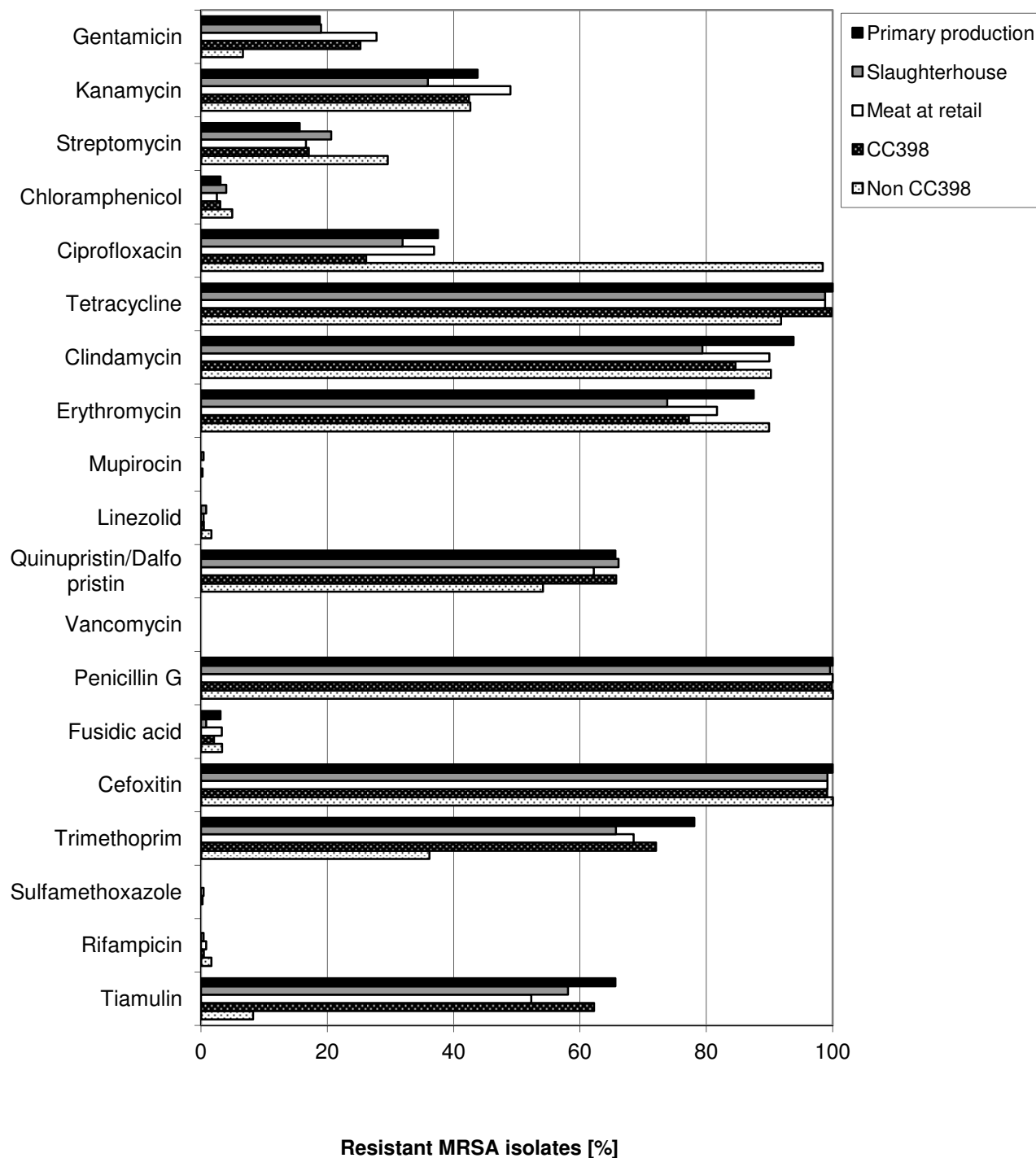


Figure 6: Antimicrobial resistance of MRSA in the German turkey meat production chain
 Distribution of antimicrobial resistance of MRSA strains separated into CC398 and non CC 398 strains as well as different steps of the turkey meat production chain isolated from dust samples at turkey primary production (n=32), carcasses at slaughter (n=248) and meat at retail (n=241). The MRSA strains were isolated in the course of the national monitoring for zoonotic agents in Germany in 2010.

Table 9: Distribution of resistance against 19 different antimicrobials grouped *spa* types and *SCCmec* types within the binary phenotypic resistance clusters of 521 MRSA isolates

The isolates were sampled at different steps of the German turkey meat production chain in 2010.

Cluster			A		B		C		total	
			n	%	n	%	n	%	n	%
No. of isolates			235	45.1	180	34.5	106	20.3	521	100
No. of resistance profiles			33	32.7	44	43.6	24	23.8	101	-
Antimicrobial substances ^a										
GEN	sen		222	55.4	176	43.9	3	0.7	401	77.0
	res		13	10.8	4	3.3	103	85.8	120	23.0
KAN	sen		150	50.0	150	50.0	0	0.0	300	57.6
	res		85	38.5	30	13.6	106	48.0	221	42.4
CHL	sen		224	44.4	178	35.3	102	20.2	504	96.7
	res		11	64.7	2	11.8	4	23.5	17	3.3
CIP	sen		152	44.6	84	24.6	105	30.8	341	65.5
	res		83	46.1	96	53.3	1	0.6	180	34.5
TET	sen		0	0.0	5	83.3	1	16.7	6	1.2
	res		235	45.6	175	34.0	105	20.4	515	98.8
CLI	sen		0	0.0	46	59.7	31	40.3	77	14.8
	res		235	52.9	134	30.2	75	16.9	444	85.2
ERY	sen		4	3.5	73	64.6	36	31.9	113	21.7
	res		231	56.6	107	26.2	70	17.2	408	78.3
MUP	sen		234	45.0	180	34.6	106	20.4	520	99.8
	res		1	100.0	0	0.0	0	0.0	1	0.2
LZD	sen		233	45.0	179	34.6	106	20.5	518	99.4
	res		2	66.7	1	33.3	0	0.0	3	0.6
SYN	sen		0	0.0	96	51.6	90	48.4	186	35.7
	res		235	70.1	84	25.1	16	4.8	335	64.3
VAN	sen		235	45.1	180	34.5	106	20.3	521	100.0
	res		0	0	0	0	0	0	0	0.0
STR	sen		189	44.5	155	36.5	81	19.1	425	81.6
	res		46	47.9	25	26.0	25	26.0	96	18.4
PEN	sen		0	0.0	1	100.0	0	0.0	1	0.2
	res		235	45.2	179	34.4	106	20.4	520	99.8
FOX	sen		2	50.0	1	25.0	1	25.0	4	0.8
	res		233	45.1	179	34.6	105	20.3	517	99.2
SMX	sen		235	45.2	180	34.6	105	20.2	520	99.8
	res		0	0.0	0	0.0	1	100.0	1	0.2
RIF	sen		234	45.2	179	34.6	105	20.3	518	99.4
	res		1	33.3	1	33.3	1	33.3	3	0.6
FUS	sen		234	45.9	175	34.3	101	19.8	510	97.9
	res		1	9.1	5	45.5	5	45.5	11	2.1
TIA	sen		0	0.0	124	53.9	106	46.1	230	44.1
	res		235	80.8	56	19.2	0	0.0	291	55.9
TMP	sen		17	10.1	144	85.7	7	4.2	168	32.2
	res		218	61.8	36	10.2	99	28.0	353	67.8

	Cluster	A		B		C		total	
		n	%	n	%	n	%	n	%
<i>Spa</i> types	t011	31	12.9	105	43.8	104	43.3	240	46.1
	t034	186	94.9	10	5.1	0	0.0	196	37.6
	other CC398	14	58.3	8	33.3	2	8.3	24	4.6
	non CC398	4	6.6	57	93.4	0	0.0	61	11.7
SCC <i>mec</i> types	III	0	0.0	4	100.0	0	0.0	4	0.8
	IVa	9	7.6	21	17.6	89	74.8	119	22.8
	V	220	65.1	108	32.0	10	3.0	338	64.9
	n.t.	6	10.2	47	79.7	6	10.2	59	11.3

^aGentamicin (GEN), kanamycin (KAN), chloramphenicol (CHL), ciprofloxacin (CIP), tetracycline (TET), clindamycin (CLI), erythromycin (ERY), mupirocin (MUP), linezolid (LZD), quinupristin/dalfopristin (SYN), vancomycin (VAN), streptomycin (STR), penicillin (PEN), ceftiofur (FOX), sulfamethoxazole (SMX), rifampicin (RIF), fusidic acid (FUS), tiamulin (TIA), trimethoprim (TMP)

The distribution of *spa* types, SCC*mec* types and antimicrobial resistance profiles within the sample collections from the three process steps and the control group were compared pair wise using the Czekanowski index (table 10). High index values were obtained for the distribution of *spa* types (PSI 0.79-0.86) among MRSA from the turkey meat chain. The comparison of the distribution of antimicrobial resistance profiles resulted in the lowest index values (PSI 0.42 – 0.56). The distribution of *spa* types and antimicrobial resistance profiles showed remarkably higher similarity between the different production steps of the turkey meat chain as to samples from the control group (PSI 0.55-0.56 and 0.13-0.19 resp.). High similarity in the distributions of SCC*mec* types was calculated between all process steps of the turkey meat production chain (PSI 0.85- 0.91). However, a strong association was also received with SCC*mec* types of the control group (PSI 0.83-0.85).

Table 10: Similarity matrix of *spa* types, *SCCmec* types and resistance profiles

The MRSA isolates originate from the German turkey meat production chain in the course of the national monitoring for zoonotic agents in 2010 (95% confidence intervals).

		Primary production	Slaughterhouse	Meat at retail	Control Group Wild boar meat
		av. PSI ^a (CI 95%) ^b	av. PSI ^a (CI 95%) ^b	av. PSI ^a (CI 95%) ^b	av. PSI ^a (CI 95%) ^b
Primary production	<i>spa</i> types	1			
	<i>SCCmec</i> types	1			
	resistance profiles	1			
Slaughterhouse	<i>spa</i> types	0.86 (0.72 , 0.95)	1		
	<i>SCCmec</i> types	0.91 (0.79 , 0.98)	1		
	resistance profiles	0.43 (0.30 , 0.53)	1		
Meat at retail	<i>spa</i> types	0.79 (0.64 , 0.90)	0.86 (0.79 , 0.92)	1	
	<i>SCCmec</i> types	0.85 (0.70 , 0.96)	0.87 (0.79 , 0.95)	1	
	resistance profiles	0.42 (0.33 , 0.51)	0.56 (0.49 , 0.62)	1	
Control Group	<i>spa</i> types	0.55 (0.33 , 0.71)	0.56 (0.38 , 0.74)	0.56 (0.38 , 0.74)	1
Wild boar meat	<i>SCCmec</i> types	0.84 (0.62 , 0.98)	0.83 (0.64 , 0.96)	0.85 (0.70 , 0.95)	1
	resistance profiles	0.13 (0.03 , 0.27)	0.19 (0.06 , 0.34)	0.14 (0.04 , 0.23)	1

^aPSI: Czekanowski index or proportional similarity index

^bCI 95%: 95% confidence interval

12.5 Discussion

In the present study, a new approach is proposed for analyzing a cross sectional set of MRSA isolates originating from three consecutive stages of the turkey meat production chain in order to draw conclusions on a potential farm to fork transmission. In the course of the German national monitoring for zoonotic agents in 2010 MRSA was isolated at all stages of the turkey meat production chain with prevalences ranging from 19.6% to 65.5%. To our knowledge, this is the first representative national MRSA prevalence study in the turkey production chain. In a regional prevalence study among fattening turkeys in southern Germany in 2009, a considerably higher prevalence of 90% MRSA positive flocks was observed using the same sampling procedure (53). The difference might be explained by the regional restriction of sampling and the small sample size in that study. The proportion of positive meat samples is in line with results from the Netherlands (15). Outside of Europe, low MRSA contamination rates of 3.85% (66) and 1.7% (7) were reported among US turkey meat.

The high MRSA prevalence in turkey carcasses after slaughter in comparison to the flock prevalence is in contrast to the situation in pigs (38, 53) and indicates that the turkey slaughter process may play an important role in the transmission of MRSA. Turkeys are slaughtered highly automated at a speed of line up to 3,600 turkey hens and up to 2,700 turkey toms per hour which leads to a permanent introduction of MRSA into the poultry processing plants (40). During the process, MRSA on animal surfaces can get transmitted via direct contact or indirect via surface processing machinery, scalding water or the hands of staff. Scalding takes place at a constant water temperature between 50 and 65°C for 60 to 210 sec (40). Although the surface of the carcasses is exposed to a heat treatment during scalding, the temperature and duration of the process might be insufficient to substantially reduce superficial MRSA counts. The selective growth of *S. aureus* after the elimination of less heat resistant microbial flora in the scalding water has been discussed (29). As bacterial counts increase in the tanks throughout the slaughter day scalding can contribute to cross contamination (26). After scalding, the birds go through the plucking machines consisting of revolving drums with rubber beaters or discs with plucking fingers. The birds are flailed and scraped for 30 – 90 sec while being sprayed with warm or cold water (40). Plucking equipment is difficult to clean and a persisting microbiological flora can get established (6). Cross contamination during slaughter and meat processing might lead to an extensive distribution of *spa* types between different animals and slaughter flocks. In addition, the increase in manual handling during processing facilitates the entry of human MRSA strains into the production units. This can explain the increase in the variability of *spa* types along the chain and is in line with the increase in the proportion of non CC398 strains in meat samples compared to dust or car-

casses. *Spa* types t002 and t1430 were also present in primary production and therefore probably have been transmitted along the food chain. In contrast, *spa* types t010, t015 were first observed in meat samples.

The majority of MRSA from the German turkey production chain was assigned to the livestock associated CC398 with the predominant *spa* types t011 and t034. This is in line with results from other livestock like veal calves (25), dairy cattle (58, 63) and pigs (20) as well as in food (15). In the present study, 37 of the 521 MRSA strains (7.1%) were identified as t002. This *spa* type t002 is assigned to CC5. In Germany, CC5 is one of the epidemic MRSA strains among humans (34). Finding t002 in turkey flocks and in turkey meat is in line with other studies from central Europe (15, 22, 53). So far, it is not known, whether this strain originates from the “human” strain and is introduced into the food chain on different levels or whether it got established in the turkey population and is transmitted along the chain. Detailed molecular-epidemiological investigations are needed to compare strains both from human and farm to fork origin. In the present study, 4.2% of the MRSA isolates were characterized as *spa* type t1430, a MRSA strain which was also frequently isolated from chicken meat (15) and broilers at slaughter (43) in the Netherlands. However, it has also been detected in turkey flocks at farm level (53). The strain is assigned to ST9, a lineage genetically unrelated to ST398. ST9 is the predominating sequence type among MRSA from pigs in Asian countries (2, 14, 37, 44, 61, 65). Outside of Europe, MRSA contamination was reported among US turkey meat (7, 66). In both surveys, all isolates belonged to USA 300 (ST8), the most common community associated MRSA strain in the USA, suggesting human contamination during processing.

The frequent use of antimicrobials at farm is discussed as a risk factor for the wide dissemination of MRSA in livestock production chains (55). In recent studies antimicrobials were identified to be used in more than 90% of the investigated turkey flocks and animals received on average 33 daily doses of antimicrobials during raising and fattening (59). With a share of 21% β -lactams were most often used followed by polypeptides (15.2%), macrolides (13.4%), tetracyclines and aminoglycosides (12.4% both). Fluoroquinolones were used in 6.5% of the investigated flocks. The common application of antimicrobials via drinking water bears the risk of under dosing of individual animals and contamination of the barn environment with antimicrobials which also facilitates the selection of resistance (52).

Cluster analysis was used to better describe the multidimensional data set of antibiotic resistance profiles grouping all MRSA strains within 3 different clusters. As the ordinal MIC values generated by two-fold dilutions in substance concentration are difficult to describe by cluster analysis a binary interpretation of the data set was used. Ward’s minimum variance with squared Euclidian distance was proven to be the best method to produce well separated cluster in binary antimicrobial resistance data sets (5, 42) No resistance phenotype simulta-

neously appeared in several clusters. The distribution of *spa* types, *SCCmec* types and the three clusters of antimicrobial resistance types did not significantly differ in the MRSA samples from the three origins. The chi square value was approaching significance with respect to the *spa* types, which was presumably due to the slightly higher proportion of other CC398 and non CC398. However, considering all three features it cannot be rejected on the basis of the included data that the MRSA isolates from different steps of the turkey meat production chain originate from the same population of strains. This result might rather indicate farm to fork transmission of MRSA of the same pool of strains than development of separate MRSA populations at each step of the chain. The calculation of the Czekanowski index for *spa* type and *SCCmec* type data results in consistently high similarity values between the matrices whereas the comparison of antimicrobial resistance phenotypes observed medium index values. Higher values of similarity were obtained between the adjacent process steps primary production/slaughter and slaughter/meat than between samples from primary production and meat. This result was expected as an increase in the variability of the MRSA isolates might be conceivable at each process stage due to external introduction of new strains via human or environmental contamination or due to spontaneous mutations in the strains.

The lower values of similarity between the distribution of *spa* types and antimicrobial resistance profiles of samples from the turkey meat production chain and the control group indicate that the proposed statistical method is valid to detect existing differences in the distribution of these characteristics of MRSA.

Concerning *SCCmec* types, high index values were also observed in comparison to the control group which might be explained by the insufficient discriminatory power of *SCCmec* typing. In addition, MRSA isolates with not typeable *SCCmec* cassettes were considered as equal that might lead to an overestimation of similarity.

It can be concluded that MRSA is present at every step of the turkey meat production chain in Germany. Using the Czekanowski index it is possible to quantify the similarity of the distribution of *spa* types, *SCCmec* types and antimicrobial resistance phenotypes between MRSA data sets from different stages of turkey meat production chain. Combined with chi square statistics, the high level of similarity suggests MRSA transmission along the chain.

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13 MRSA in cattle food chains

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Bernd-Alois Tenhagen, Birgit Vossenkuhl, Annemarie Käsbohrer, Katja Alt, Britta Kraushaar, Beatrice Guerra, Andreas Schroeter and Alexandra Fetsch

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The approach chosen in the paper is based on the work of chapter 11. Birgit Vossenkuhl performed the calculation of the proportional similarity index (PSI), wrote the respective parts of the manuscript and was engaged in critical reading and revision of the manuscript.

13.1 Abstract

Livestock associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) have been found in various farm animal species throughout the world. It was the objective of this study to estimate the prevalence of MRSA in different cattle food chains (milk, beef, veal) in Germany, to analyse the MRSA diversity along each food chain and to compare the characteristics of the different subtypes. Samples were collected between 2009 and 2012 from dairy herds (bulk tank milk), veal herds (dust from the stables), veal calves and beef cattle at slaughter (nasal swabs), carcasses of veal calves (surface cuts) and beef as well as veal at retail. Sampling was proportionally distributed over the country according to the cattle population (on farm sampling), slaughterhouse capacity (abattoir samples) and the human population (meat at retail). MRSA were isolated using harmonized methods from all sample types and populations investigated. The highest proportion of positive samples was found in nasal swabs from veal calves at slaughter in 2012 (144/320, 45.0 %), the lowest rate in bulk tank milk in 2009 (14/388, 4.1 %). Most isolates, irrespective of the origin, were from *spa* types t011 and t034. Both have been assigned to the clonal complex (CC)398. Few isolates (15/632; 2.4 %) were from *spa* types not associated with the CC398. *Spa* type patterns were similar along individual food chains, but differed between food chains. Antimicrobial resistance patterns differed between isolates from the different food chains and *spa* types. Isolates from the veal chain displayed the highest resistance rates. We conclude that there is substantial diversity in the MRSA prevalence across different cattle production sectors.

Key words: antimicrobial resistance, cattle, food chain, methicillin, *Staphylococcus aureus*,

13.2 Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) have been frequently detected in livestock in recent years. In cattle, first reports on MRSA date back to the 1970es, describing individual isolates from cases of mastitis in dairy cows (12). In 2007, a report on transmission of MRSA between dairy cows and milking personal alerted people working with dairy cattle of the occupational health risks (27). In the following years a number of reports have described the prevalence of MRSA in dairy cattle and transmission of MRSA between people working on farms and dairy cattle (3; 18; 22; 32; 41). Likewise, calves on dairy farms were found positive for MRSA (41). Only 1 study, testing a very small milk sample per herd failed to detect MRSA (46). A comparatively high prevalence of MRSA was found in veal calves (20), while beef cattle in feedlots were tested negative in Canada (49).

S. aureus is one of the leading causes of foodborne outbreaks due to its ability to produce staphylococcal enterotoxins (23). Recently, a study conducted in Canada did not find any MRSA among *S. aureus* isolates involved in staphylococcal food poisoning outbreaks (10). This is in line with the absence of enterotoxin genes in MRSA from bulk tank milk or bovine meat (4; 28). So far, livestock-associated MRSA (LA-MRSA) are not considered to be transmitted via the ingestion of food.

This report describes the results of the investigations into MRSA in cattle from farm to fork, including dairy cattle, veal calves, beef animals, and food thereof. Our hypotheses were that

1. MRSA prevalence differs between production systems;
2. MRSA from dairy herds and veal calves are similar, as veal calves are frequently born in dairy herds;
3. MRSA in meat mainly originate from primary production.

13.3 Materials and Methods

13.3.1 Sampling

In Germany, a monitoring system for zoonotic bacteria in the food chain has been established in 2009 to fulfill the requirements of Directive 2003/99/EC (2; 16). The general aim of the monitoring system is to investigate the prevalence of zoonotic bacteria along the different food chains and to collect isolates of the different bacterial classes for further characterization, e.g. typing and antimicrobial resistance testing.

Sampling plans were designed to cover primary production in dairy cattle, beef cattle, veal calves and meat at retail. Milk at retail was not included in the studies as milk is heat treated before being sold to the consumer with very few exceptions. Therefore, transmission of MRSA to milk at retail was not investigated. The exceptions were covered by a study on MRSA in bulk tank milk from dairy herds certified for marketing of raw milk to consumers. Within the monitoring system sampling plans are designed annually for collecting samples at farm, at the abattoir and from food at retail. Sampling plans in the German system are negotiated between the federal institutions and the regional authorities to assure a high degree of compliance with the decided sampling procedures. This procedure has been fixed in national legislation (2). Sampling at farm was distributed across the federal states proportionally to the number of animals kept. Sampling frequency at the abattoir was guided by the annual throughput of the abattoirs with respect to the animal category tested. Sampling at retail was proportional to the human population of the federal state as the focus here was on exposure of humans to MRSA via meat. Sample size was estimated as previously reported (25) based on an estimated prevalence of 50 % as prior knowledge was not fully available. The numbers of samples taken per category are given in table 11.

On dairy farms, bulk tank milk samples were collected (1 sample per farm per year). Dairy farms included randomly chosen conventional dairy farms with at least 20 lactating cows. In 2010, 30 so called certified farms were additionally included. These farms are allowed to sell raw milk to consumers (“Vorzugsmilch”) but have to take additional hygienic measures in comparison to conventional farms (1). Regional authorities were advised to collect samples from all the farms of this type in their region.

On veal calf farms, five dust samples were collected from different surfaces of the stable and were pooled for analysis. Veal calves are typically raised to the age of 8 months mostly on liquid feed (milk or milk replacer) to produce veal. In 2012, veal calf herds and farms housing animals up to 12 months were included to be in line with recent recommendations from the proportional similarity index (EFSA) (14).

At the abattoir, nasal swabs were collected from 1 animal per slaughter batch and excision samples (1 per slaughter batch) were collected from carcasses of veal calves and young cattle up to the age of 12 months. In 2011, beef animals were sampled. Those are typically 18 to 30 months old at slaughter and may have been raised under intensive conditions (confined housing for the complete lifetime) or under semi-intensive conditions (free range housing with suckler cows up to weaning and confined housing thereafter).

At retail, beef, veal and meat preparations from veal were sampled. Food items covered by the inclusion criteria were sampled. Sampling personnel made sure that only one sample per production batch was collected.

Samples were collected by veterinary officials of the federal states and transported to the laboratory in cooled containers with the exception of dust samples that did not have to be cooled. MRSA were isolated from the samples by the regional laboratories of the individual federal states according to pre-described methods.

13.3.2 Isolation of MRSA

Regional laboratories were provided with a standard method recommendation for the isolation of MRSA by the National Reference Laboratory for coagulase positive staphylococci including *S. aureus* (NRL-Staph) at the Federal Institute for Risk Assessment (BfR). The five dust samples were pooled per farm in 100 ml Mueller Hinton broth supplemented with 6.5% (6.0 %) NaCl for pre-enrichment (MHB+). Milk samples (25 ml), fresh meat (25 g) and meat preparations (25 g) were pre-enriched in 225 ml MHB+. After incubation for 16-20 h at 37°C, 1 ml pre-enrichment broth was transferred into 9 ml of tryptic soy broth supplemented with 3.5 mg/l cefoxitin and 75.0 (50.0) mg/l aztreonam, respectively. In January 2011, the 2 enrichment broths were slightly modified following an internal evaluation process (unpublished data). Salt content of MHB was slightly reduced (from 6.5 to 6.0 %). Likewise, the aztreonam content of the tryptic soy broth was reduced from 75 to 50 mg/l. After incubation of this selec-

tive-enrichment broth for a further 16-20 h at 37°C one loopful was plated onto chromogenic MRSA screening agar, and incubated for 24-48 h at 37°C. Presumptive MRSA isolates were sent to the 'NRL-Staph' for confirmation, typing and further analysis. The number of MRSA isolates included in further analyses is not exactly congruent to the number of positive samples obtained within the national monitoring because first, the NRL did not always receive the corresponding isolate from the regional laboratories second, isolates which did not exactly correspond to the monitoring sampling plan but were from the target population were excluded from prevalence estimations but included in further typing and resistance testing.

13.3.3 Confirmation and Molecular typing

Presumptive MRSA isolates were confirmed by an in-house multiplex PCR simultaneously targeting the 23S rDNA specific for *Staphylococcus* species, the nuclease gene *nuc* which is specific for *S. aureus*, and the resistance gene *mecA* (36). Template DNA was extracted from isolates using commercial kits ("RTP® Bacteria DNA Mini Kit", Invitex, Berlin, Germany, "DNeasy Blood and Tissue kit", Qiagen, Hilden, Germany). All MRSA isolates were further characterized using *spa* typing (40) and SCC*mec* typing (50), the latter differentiating between SCC*mec* types I to V, including V*(5). The software Ridom Staphytype (Ridom GmbH, Würzburg, Germany) was used to assign *spa* types. *Spa* types that had not been identified and assigned to a clonal complex (CC) by the NRL before were additionally subjected to multilocus sequence typing (MLST) (15).

13.3.4 Antimicrobial susceptibility testing

All isolates were tested for the susceptibility to antimicrobials using the broth microdilution method in accordance with Clinical and Laboratory Standards Institute guidelines (9). Commercial microtitre plates were used (TREK Diagnostic Systems, Magellan Biosciences, West Sussex, England). Evaluation of the minimum inhibitory concentrations (MIC) was based on epidemiological cut-off values (ECOFF) published by the European committee for antimicrobial susceptibility testing for MRSA and/or *S. aureus* (17). MIC values above the ECOFF indicated microbiological resistance. MIC values lower or equal to the ECOFFs characterised susceptible strains. *S. aureus* strain ATCC 25923 was used for quality assurance. The following antimicrobials were tested (ECOFF (mg/l) in brackets): gentamicin (≤ 2), kanamycin (≤ 8), chloramphenicol (≤ 16), ciprofloxacin (≤ 1), tetracycline (≤ 1), clindamycin (≤ 0.25), erythromycin (≤ 1), mupirocin (≤ 1), linezolid (≤ 4), vancomycin (≤ 2) and the combination of quinupristin and dalfopristin (≤ 1).

13.3.5 Statistical analysis

Statistical analyses were carried out using PASW Statistics (Version 18.02, IBM Deutschland, Ehningen, Germany) and the open source software “R”. Prevalence estimates of MRSA were compared by simple chi-square test where appropriate. Although not all isolates were available for confirmation at the NRL, all samples reported positive by the regional laboratories were considered positive for the prevalence estimates. Prevalence of MRSA was only compared if the same kind of samples was collected at the same stage of the food chain.

Spa types were categorized in 4 different categories. Types t011 and t034 were 2 separate categories, as they were identified in most isolates. Other *spa* types that have been assigned to CC398 were categorized together as “other CC398”. The fourth category consisted of those isolates that were not assigned to CC398 and named non CC398.

Antimicrobial resistance (AMR) in MRSA was analyzed using logistic regression by substance. Only substances showing differences in resistance rates of more than 20 % between isolates of different sources or isolates of different *spa* types were included in the testing. In the logistic regression model the outcome considered was resistant (1) or non-resistant (0). Food chain (dairy vs. beef vs. veal), *spa* type group and SCC*mec* type were included as categorical covariates.

The degree of similarity between the frequency distributions of *spa* types of MRSA among the sample sets from the cattle food chains was estimated using the Czekanowski index or proportional similarity index (PSI) (38). It is calculated by:

$$PS = 1 - 0.5 \sum_i |p_i - q_i| = \sum_i \min(p_i, q_i)$$

where p_i and q_i represent the proportion of strains out of all strains among the data sets P and Q which agree in the realization i of the variable of interest. The values for PS range from 1 for identical frequency distributions of the variable of interest to zero for no similarities between the data sets. Since the size of the samples is rather small, a realization of the PSI index may deviate largely from its true value. Thus, the PSI was bootstrapped obtaining a probability density distribution from which we derived the 95% confidence interval for the PSI. The statistic open source software R was used to calculate the approximate confidence interval of the Czekanowski index using the bootstrap method utilizing 1000 iterations (13).

13.4 Results

13.4.1 Prevalence

MRSA were detected in all types of samples taken (table 11). Prevalence was highest in veal calves. Herd level prevalence in 2010 and 2012 was identical, the prevalence in nasal swabs at the abattoir increased from 2009 to 2012. Prevalence in nasal swabs from beef cattle at slaughter was substantially lower. In dairy cows, herd level prevalence was similar in both years (2009, 2010). It was numerically higher in the samples from certified farms, but the number of samples was low and therefore the difference was not significant.

Table 11: Prevalence (and 95% CI) of MRSA in samples of different cattle food chains in Germany (2009 to 2012)

Food chain	Sample type		2009	2010	2011	2012
Dairy cattle	Bulk tank milk, conventional farms	No ¹	14/338	14/297		
		% (95 % CI)	4.1 (2.0-6.3)	4.7 (2.3-7.1)		
	Bulk tank milk, certified farms ¹	No		3/30		
		%		10.0 (0-20.7)		
Veal calves ²	Dust samples ³	No		58/296		46/240
		%		19.6 (15.1-24.1)		19.2 (14.7-24.6)
	Nasal swabs at slaughter	No	123/350			144/320
		%	35.1 (30.1-40.1)			45.0 (39.6-50.5)
	Carcass at slaughter	No				96/312
		%				30.8 (25.9-36.1)
Veal at retail	No	48/387			44/421	
	%	12.4 (9.1-15.7)			10.5 (7.9-13.8)	
Beef animals	Meat preparations with veal	No	6/31			
		%	19.4 (5.4-33.3)			
	Nasal swabs at slaughter	No			25/288	
		%			8.7 (5.9-12.5)	
Beef at retail	No			41/509		
	%			8.1 (6.0-10.8)		

¹ No of positive samples / No of samples

² farms producing certified milk (Vorzugsmilch) according to German law (1)

³ in 2009/2010 cattle up to the age of 8 months were included, in 2012 cattle up to the age of 12 months were included.

⁴ Numbers refer to (positive) pools of sample

13.4.2 Typing results

A total of 632 isolates were confirmed as MRSA at the NRL-Staph (table 12). Overall, 28 different *spa* types were identified among these isolates. *Spa* types t011 (58.1 %) and t034 (32.0 %), both assignable to CC398, predominated with a combined proportion of 90.0 % of all isolates tested, ranging from 83.3 to 96.6 % per sample type and year. Other *spa* types were also mostly assignable to the clonal complex CC398 (7.6 %; range 0 to 16.7 %). Non CC398 *spa* types were rare (15 isolates, 2.4 %) and mostly identified in retail meat (12/15 isolates, 8.3 % of the 142 isolates from meat). Only 3 of the 490 isolates that did not originate from retail meat were non CC398 (0.6 %). Those were identified as t002, t009 and t1919 and were isolated from herds of veal calves at farm (2 isolates) or veal calves at slaughter (1 isolate).

Diversity of MRSA tended to be minimal in bulk milk tank samples that harbored only 3 different *spa* types (29 isolates). In contrast, 11 different *spa* types were isolated from dust samples from veal farms, veal calves at slaughter and from veal at retail (table 12).

Table 12: Proportion of the different *spa* types in the individual sample categories

Clonal Complex	<i>Spa</i> type	Veal calf			Beef cattle		Dairy cattle		Total
		Dust sample at farm	Nasal swab at slaughter	Carcass at slaughter	Veal at retail	Nasal swab at slaughter	Beef at retail	Bulk tank milk	
CC398	Total (No)	81	260	90	97	27	33	29	617
	t011 (%)	51.8	59.4	50.0	57.3	77.8	64.1	65.5	58.1
	t034 (%)	36.1	34.1	33.3	31.1	14.8	20.5	31.0	32.0
	t1197 (%)	1.2	1.5	5.6					1.6
	t108 (%)	1.2	0.4	4.4	1.9				1.3
	t2346 (%)		1.9	2.2		3.7			1.3
	t1451 (%)	2.4	0.4						0.5
	t6325 (%)		0.8	1.1					0.5
	t899 (%)	1.2	0.4	1.1					0.5
	t1456 (%)	1.2			1.0				0.3
	t571 (%)		0.4			3.7			0.3
	t10890 (%)		0.4						0.2
	t11614 (%)				1.0				0.2
	t1255 (%)	1.2							0.2
	t1457 (%)							3.4	0.2
	t2123 (%)			1.1					0.2
	t2383 (%)				1.0				0.2
	t2510 (%)			1.1					0.2
	t4652 (%)				1.0				0.2
	t5210 (%)	1.2							0.2

Clonal Complex	<i>Spa</i> type	Veal calf			Beef cattle		Dairy cattle		Total
		Dust sample at farm	Nasal swab at slaughter	Carcass at slaughter	Veal at retail	Nasal swab at slaughter	Beef at retail	Bulk tank milk	
non CC398	Total (n)	2	1	0	6	0	6	0	15
	t002 (%)	1.2			1.9				0.5
	t1430 (%)				1.9		2.6		0.5
	t008 (%)						5.1		0.3
	t127 (%)				1.0		2.6		0.3
	t009 (%)	1.2							0.2
	t1419 (%)				1.0				0.2
	t1919 (%)		0.4						0.2
	t283 (%)						2.6		0.2
	t3276 (%)						2.6		0.2
No of isolates		83	261	90	103	27	39	29	632
No of different <i>spa</i> types		11	11	9	11	4	7	3	28

Three different specific *SCCmec* types were identified. *SCCmec* V was the most frequent type with 75.3 % of the isolates. Type IVa was the second most frequent (18.5 %) and type V* the least frequent (3.0 %). Some isolates were not typeable (3.0 %).

SCCmec type V was most frequent in all *spa* types assigned to CC398 (figure 7). Type IVa occurred frequently in t011 and other CC398 but was rare in t034. It was the most frequent type in the non CC398 isolates. *SCCmec* type V* was mainly observed in t034 isolates (19/20 type V* isolates) where it accounted for 9.6 % of all isolates. Of the 20 isolates that were not typeable concerning their *SCCmec* type, 14 were *spa* type t011, the others were non CC398 isolates.

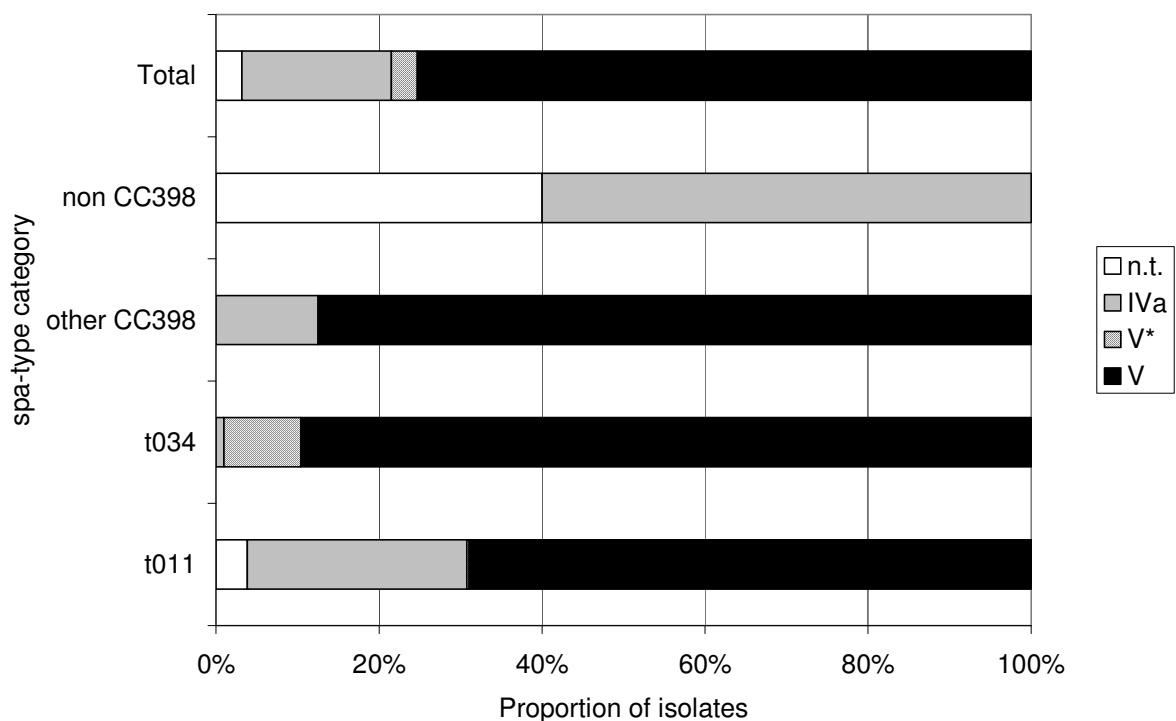


Figure 7: Proportion of the different *SCCmec* types in the different *spa* type categories

13.4.3 Similarities between *spa* type patterns at the different stages of the food chain.

Overall, *spa* type patterns were similar within the same food chain (table 12). Most of the isolates found in nasal swabs of veal calves at slaughter were from *spa* types that had also been isolated from dust on veal calf farms (251/261, 96.2 %). Likewise, isolates found on carcasses mostly were from *spa* types that were also found in nasal swabs (97.8 %). In veal

at retail, 10 % of the isolates were from *spa* types that had not been identified in carcass swabs. Moreover, 7 of these 10 isolates were from *spa* types that were not identified in any other sample from the veal food chain.

In beef, none of the non CC398 associated *spa* types were identified in nasal swabs at slaughter. Figure 8 displays the quantification of the similarity of the *spa* type patterns in the veal food chain using the PSI. The index was fairly high for all pairs analyzed. The index was highest between the isolates from nasal swabs from veal calves at slaughter and those from the carcasses sampled in the same year (0.89, 95 % CI 0.79-0.97) and between dust samples on farm and nasal swabs at slaughter sampled in the same year (0.86, 95 % CI 0.69-0.97). It was somewhat lower when isolates from meat at retail were compared with those from primary production or at slaughter. It was also lower for the comparison of isolates from 2 different sampling years, i.e. 2009 and 2012.

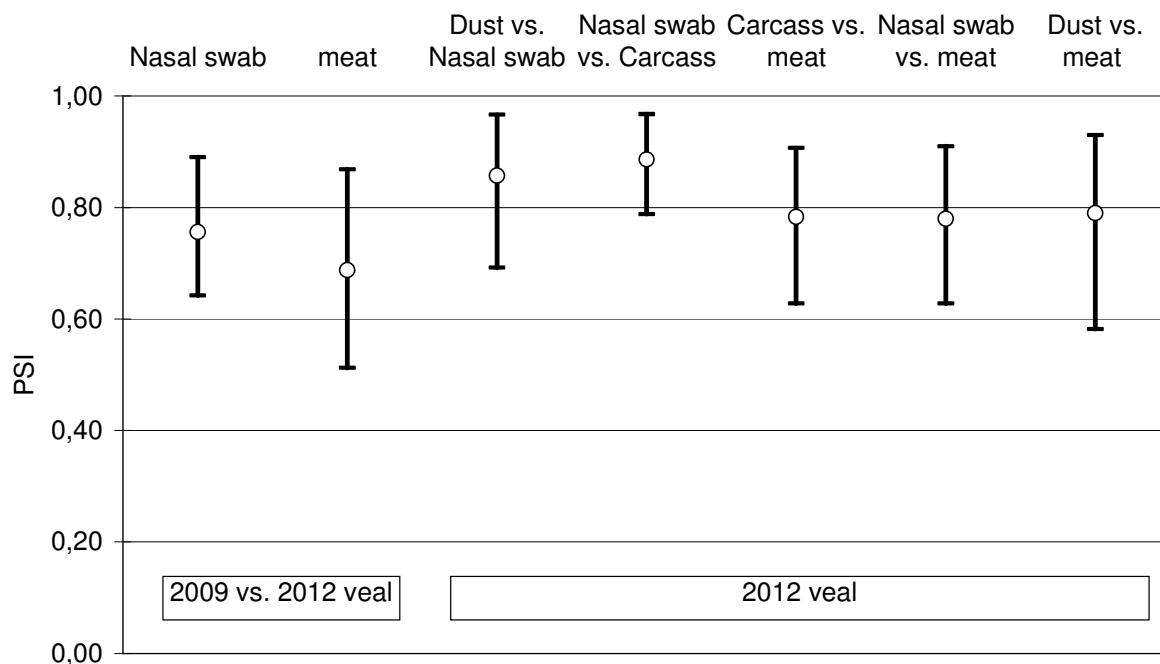


Figure 8: Proportional similarity index (PSI, ○) and confidence intervals (error bars) for *spa* types of isolates from different years and from different stages of the veal food chain

13.4.4 Antimicrobial resistance (AMR)

Of the 632 isolates tested only 1 isolate was not resistant to any further antimicrobial than beta-lactams. Antimicrobial resistance varied between the 3 food chains with the veal food chain showing the highest number of resistances in the isolates (median 5 substances vs. 3 in beef chain and 4 in dairy cattle, $p < 0.01$).

Considering the individual substances, 5 of the 11 substances showed minimal variation between food chains and subtypes of MRSA because either nearly all isolates were susceptible (chloramphenicol, mupirocin, linezolid, vancomycin) or most isolates were resistant (tetracycline) (table 13). Only some non CC398 isolates were susceptible to tetracycline (4/15, 26.7 %).

Table 13: Antimicrobial resistance (%) in MRSA isolates from different stages of different cattle food chains 2009-2012

Antimicrobial	Veal calf, dust	Veal calf, nasal swab	Veal calf, carcass	Veal	Veal chain, total	Beef cattle nasal swab	Beef	Beef chain, total	Bulk tank milk	Total
No of isolates	83	261	90	103	537	27	39	66	29	632
Gentamicin	25.3	37.5	33.3	21.4	31.8	3.7	17.9	12.1	24.1	29.4
Kanamycin	41.0	49.8	44.4	35.0	44.7	11.1	38.5	27.3	34.5	42.4
Erythromycin	75.9	69.3	70.0	60.2	68.7	29.6	64.1	50.0	51.7	66.0
Clindamycin	81.9	78.5	74.4	68.0	76.4	37.0	59.0	50.0	62.1	72.9
Chloramphenicol	7.2	5.4	6.7	5.8	6.0	7.4	7.7	7.6	3.4	6.0
Tetracycline	100.0	100.0	100.0	99.0	99.8	100.0	92.3	95.5	100.0	99.4
Ciprofloxacin	18.1	14.6	10.0	10.7	13.6	7.4	17.9	13.6	0.0	13.0
Synercid	48.2	44.4	46.7	37.9	44.1	29.6	35.9	33.3	27.6	42.2
Mupirocin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Linezolid	1.2	0.8	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.5
Vancomycin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Further statistical analyses were restricted to the other 6 substances (table 14). Significant differences in the resistance rates between the food chains were observed for gentamicin, kanamycin, erythromycin and clindamycin. In all cases the odds of resistance to the respective substance were lower for isolates from the beef chain compared to those from the veal chain. No significant difference was observed between the resistance rates in isolates from dairy cattle chain and the other chains.

Spa types were associated with AMR to all the 6 substances (figure 9). *SCCmec* type was associated to resistance against 5 of the 6 substances (all except ciprofloxacin). Interestingly, all significant associations indicated that *SCCmec* type V was less likely resistant than the other less frequent *SCCmec* types.

Three odds ratios were not calculated as either all or none of the isolates were resistant. None the 29 dairy cattle isolates was resistant to ciprofloxacin, while 13.6 % of the isolates from the beef and the veal food chain were resistant to this fluoroquinolone (Table 13). *SCCmec* type V* was consistently susceptible to gentamicin and ciprofloxacin.

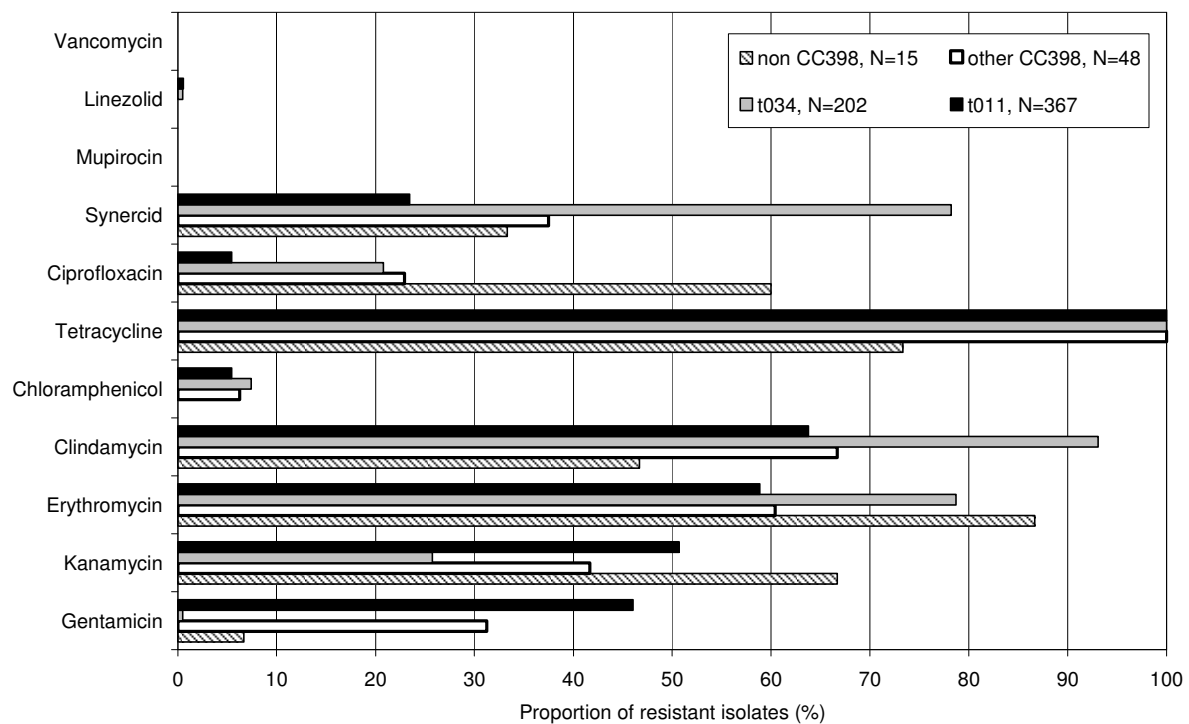


Figure 9: Antimicrobial resistance in isolates from different *spa* type categories (n=632)

Table 14: Association of antimicrobial resistance to selected substances, typing results and food chain (n=632).

Results of logistic regression for each substance including food chain, *spa* type and *SCCmec* type as covariates. Significant associations are depicted in bold. “veal”, “t011” and “*SCCmec* type V” were the reference categories for all analyses.

Food chain	OR	CI-	CI+	<i>Spa</i> category	OR	CI-	CI+	<i>SCCmec</i> type	OR	CI-	CI+
Veal	Reference			t011	Reference			V	Reference		
Gentamicin											
Beef	0.32	0.14	0.75	t034	0.01	0.00	0.07	IVa	31.10	14.08	68.72
Dairy	0.45	0.13	1.51	Other CC398	0.64	0.30	1.35	Not typeable	0.86	0.26	2.86
				Non CC398	0.01	0.00	0.11	V* ¹	Not feasible		
Kanamycin											
Beef	0.49	0.25	0.94	t034	0.48	0.31	0.75	IVa	34.07	14.19	81.83
Dairy	0.53	0.20	1.37	Other CC398	0.90	0.45	1.78	Not typeable	0.79	0.26	2.39
				Non CC398	0.96	0.17	5.26	V*	5.55	2.11	14.63
Erythromycin											
Beef	0.52	0.30	0.90	t034	2.79	1.82	4.28	IVa	2.79	1.82	4.28
Dairy	0.48	0.22	1.05	Other CC398	1.09	0.58	2.06	Not typeable	1.09	0.58	2.06
				Non CC398	5.33	0.99	28.58	V*	5.33	0.99	28.58
Clindamycin											
Beef	0.43	0.24	0.77	t034	8.76	4.76	16.13	IVa	2.09	1.25	3.49
Dairy	0.51	0.22	1.19	Other CC398	1.21	0.63	2.32	Not typeable	8.42	1.83	38.83
				Non CC398	0.21	0.06	0.76	V*	1.98	0.24	16.21

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Food chains	OR	CI-	CI+	<i>Spa</i> category	OR	CI-	CI+	SCC <i>mec</i> type	OR	CI-	CI+
Ciprofloxacin											
Beef	0.86	0.36	2.04	t034	4.11	2.25	7.50	IVa	0.34	0.11	1.06
Dairy ¹	Not feasible			Other CC398	4.39	1.91	10.06	Not typeable	0.73	0.13	4.23
		Non CC398		Non CC398	44.94	9.82	205.74	V* ¹	Not feasible		
Synercid											
Beef	0.91	0.48	1.73	t034	12.11	7.68	19.08	IVa	0.86	0.50	1.48
Dairy	0.42	0.16	1.16	Other CC398	2.12	1.11	4.05	n.t.	12.74	3.85	42.12
				Non CC398	0.54	0.12	2.42	V*	2.20	0.58	8.35

¹No OR calculated as all isolates were susceptible

13.5 Discussion

This is the first description of results of representative studies on MRSA along several cattle food chains of a country. The results show that, in Germany, MRSA can be found in dairy, beef and veal production systems including the meat and milk produced from animals raised in these systems. Concerning dairy cattle and veal calves the results confirm other studies that were published previously (18; 19; 41; 45). Studies in beef animals are rare so far. A Canadian study did not detect MRSA in feedlot cattle (49). MRSA in beef had been reported previously from the Netherlands and the US, albeit at low proportions (11; 24). At the same time, several studies failed to detect MRSA in beef (8; 21).

13.5.1 Prevalence and typing results

Results for carcasses at slaughter as well as the similarity between *spa* type patterns in the environment of the animals and their nasal swabs, their carcasses and meat thereof indicate that MRSA are readily transmitted to the carcass during slaughter and also further down the food chain during processing. Veal calves mostly originate from dairy herds. Therefore, MRSA in the calves may originate from the dairy production system. This is in line with the results of our study, as 2 of the 3 *spa* types that were identified in bulk tank milk were also observed in veal calves. The third *spa* type (t1457, 1 isolate) was neither observed in beef nor in veal animals. However, as it was infrequent in dairy herds it may have escaped detection in the veal or beef herds.

The prevalence of MRSA in dairy herds was based on bulk tank milk samples. Recently, an Italian study carried out in herds that were suspected to be MRSA positive indicated that bulk tank milk samples may underestimate the prevalence of MRSA in dairy herds. However, the authors used a selective broth with much higher levels of antimicrobials and did not report the amount of milk included in the sample (3). It is not clear whether this may have hampered sensitivity of bulk tank milk analysis.

The diversity of *spa* types was higher in veal calves than in dairy cattle. This has been explained by the diverse origin of the calves raised on veal farms. For the veal industry of the Netherlands it has been reported that their calves originated from a number of different EU-Member States (48). In contrast to pigs or poultry, where sows and hens produce 25 piglets and more than 200 chicks per year, cows usually have 1 calf. Therefore, the number of calves born on a dairy farm is limited. Veal calf herds need to purchase animals from a great variety of farms or through markets or traders. On the one hand this increases the risk that at least one of the calves originates from a MRSA-positive dairy farm. On the other hand, these veal farms frequently use antimicrobials to counteract disease conditions associated with

crowding, hence MRSA introduced by individual positive calves are exposed to highly favourable conditions of selection pressure towards antimicrobial resistances (34; 35).

The proportion of MRSA positive beef animals at slaughter was substantially lower than that observed for veal calves. Animals at slaughter do not exactly reflect the situation on farm as bacterial colonization may also have been acquired during transport or in the lairage facilities as reported for MRSA in pigs (7). However, transport and lairage are factors that all large slaughter animals are exposed to. Therefore, the difference observed in the prevalence at slaughter may indicate a similar difference in primary production which is in line with the observed data on MRSA in veal calves at farm. A different level of antimicrobial use between beef and veal animals could have contributed to the differences. Differences in the level of antimicrobial use have recently been reported for Lower Saxony, the German federal state housing a substantial part of the German veal industry (34).

The rate of positive veal carcasses was high as compared to data available for pigs (6; 26). The reason for this remains to be elucidated. In pigs, comparatively low detection rates on carcasses were explained by heat treatments applied to the carcass surfaces during the slaughter process (30). Such treatments are not applied to cattle. However, as the skin is removed a massive reduction of the contamination could have been expected and has been reported with respect to verotoxigenic *E. coli* (43). It is not clear, why with respect to MRSA, no such reduction occurs. In contrast to Verotoxin producing *E. coli* (VTEC), MRSA is not an enteric pathogen and therefore fecal recontamination is not a likely source of the isolates on the surface of carcass. A potential role of contaminated slaughter equipment needs to be investigated as *S. aureus* is well known for its ability to form biofilms (29). Moreover, aerosols associated with the mechanic removal of the skin could be involved in the contamination of carcasses (39). Slaughterhouse personnel may be involved in the transmission, however, they are not a likely source of MRSA as all of the MRSA on the carcasses were from the clonal complex CC398 that is still infrequent in the human population and in slaughter personnel that does not handle live animals (33; 44).

In retail meat, diversity of strains was high. Veal and beef at retail not necessarily is derived from domestic production and divergent strains may simply reflect a different origin. However, most of the isolates were from *spa* types that had also been observed in primary production and animals at slaughter which supports the hypothesis that MRSA in meat from cattle mainly originate from primary production. The comparatively high proportion of non CC398 strains in meat at retail (8.3 %) and the lower PSI observed when comparing meat at retail with carcasses or animal samples suggests that additional clones of MRSA are transmitted to meat that probably do not originate from primary production but from people handling the meat during processing or at retail. Yet, compared to the CC398 strains, the proportion is

comparatively small and primary production therefore can be considered the main source of MRSA on retail meat.

Traded slaughter animals may not be an explanation as MRSA in veal calves in the Netherlands are also mainly from clonal complex CC398 (19).

In contrast to the situation in turkey meat the non CC398 MRSA found in beef and veal do not belong to 1 or 2 other distinct clonal complexes but are more diverse. In the turkey meat food chain it could be shown that most of the non CC398 strains occurring in meat were from 2 distinct *spa* types, i.e. t002 (CC5) and t1430 (CC9) that were also frequently found in primary production (47).

The association of the 2 *spa* types t011 and t034 with certain SCC*mec* types has been reported before. In a study in slaughter pigs in Germany, most of the isolates harbouring SCC*mec* type V* were from *spa* type t034 (94.0 %) (42). The similarity of this pattern indicates that the same MRSA clones that spread in the pig population in Germany can also be found in the cattle population. However, in-depth molecular-biological analyses are needed to confirm this hypothesis.

13.5.2 Antimicrobial resistance

Antimicrobial resistance was high in the MRSA isolates from all sample types, but highest in veal calves. This adds to the observed higher frequency of MRSA in the veal calf chain. This is not surprising given the massive exposure of veal calves to antimicrobials (35; 48). Although intensively housed beef cattle are also frequently exposed to antimicrobials exposure is substantially lower than in veal calves (34)

As previously described for LA-MRSA from animal origin in Germany (4; 42), resistance to tetracycline was common with only few isolates susceptible to this antimicrobial. Likewise, resistance to clindamycin and erythromycin was widespread. However, resistance to these antimicrobials was significantly higher in the veal chain than in the beef chain. The same applied for resistances to gentamicin. Aminoglycosides, macrolides and lincosamides are commonly used in veal calves but also in beef cattle (34), although less frequently. Resistance of isolates from dairy cows was numerically lower than those from veal and beef cattle, but due to the low number of isolates from bulk tank milk the differences were not significant.

Antimicrobial resistance was also associated with *spa* types. This has been observed before (42). The reasons for the differences in the resistance patterns of the different *spa* types are not clear. t011 and t034 differ substantially with respect to AMR although both *spa* types were frequent in all sample materials. Recently, t034 clustered separately in a study using whole genome sequencing (37). This indicates that t034 is probably a distinct clone that dif-

fers substantially from t011 although the *spa*-repeat patterns are very similar. This adds to the difference observed with respect to the SCC*mec* types.

Resistance patterns differed between non CC398 isolates and CC398 isolates. A lower resistance rate to tetracycline and a higher resistance rate to ciprofloxacin indicate that there might be human associated strains among these isolates, as ciprofloxacin resistance is typical for hospital-acquired-MRSA and resistance to tetracycline is infrequent in these isolates (31). Again, these findings call for in-depth molecular comparison of the strains.

13.6 Conclusions

MRSA prevalence differs between the 3 cattle production systems compared, with the veal chain displaying the highest prevalence. Most of the isolates from veal calves are from the same *spa* types observed in dairy herds, however, overall diversity seems to be higher in calves. MRSA in meat (veal and beef) are very similar to those for primary production indicating transmission of the bacteria along the food chain. However, data also indicate that further MRSA clones of potentially human origin may be introduced into the cattle food chains during processing.

13.7 Reference List

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14 General discussion

Several individual investigations have proven evidence that livestock associated MRSA are present at any key step of the production chain of economically important meat species including pork, poultry and beef. Farm to fork transmission has been assumed previously. However, no approach has been proposed for evaluating potential MRSA transmission along the food chain which exceeds the level of a merely descriptive depiction of MRSA prevalence and typing data. In the present thesis, new methodological concepts have been developed which are appropriate to demonstrate MRSA transmission along the food chain.

14.1 MRSA transmission along the pork supply chain

Since LA-MRSA had been firstly described in 2004 it soon became evident that the pig primary production is one of its most important reservoirs (39). Since then, an increasing number of investigations reported that LA-MRSA is not only highly prevalent among pigs at farm level but can also be isolated from subsequent process steps of the pork supply chain as well as from pork (2, 12, 35). In order to evaluate the burden of MRSA in the pork production sector a comprehensive literature review was conducted. For this purpose, scopus <http://www.scopus.com> and <http://www.pubmed.com> were searched using the keywords MRSA and *Staphylococcus aureus* in combination with ST398, CC398, pig, meat, food, slaughter, hygiene or hospital. In addition, listed references of the studies were cross-checked. Primary research articles which provide prevalence and typing data of MRSA on the process steps pig primary production, transport, slaughter, processing and final pork product were included into the review. MRSA prevalence data were extracted and summarized at country level separated by the process steps primary production, slaughter and pork. The appearance of dominant genetic variants was compared likewise. The summarization of risk factors for the within herd and between herd transmission at primary production level were summarized. A detailed analysis of the pig slaughter process with special emphasis on the changing prevalence of different microorganisms along the chain was used to draw conclusions about critical steps for MRSA transmission.

The literature review could confirm that LA-MRSA is widely spread in the pig supply chain. LA-MRSA can be isolated from all key steps of the pork production chain including meat worldwide. The prevalences vary greatly depending on region and process step. Animal age, herd size and the type of animal replacement policy followed on farm significantly influence the spread of MRSA within and between pig herds. Furthermore, the individual MRSA detection rate was shown to correlate with the pig density of the region under study and the type of

pig farm. The correlation between the use of antimicrobials and the prevalence of MRSA in pig husbandry was assumed repeatedly (2, 7, 8, 17) but has only recently been confirmed for groupwise antimicrobial treatment during the fattening period (18).

The comparative compilation of typing data revealed regional specific distribution patterns of dominating genetic LA-MRSA variants which could be retrieved on each stage of the production chain of the respective country. LA-MRSA had always been present at former stages of the pork supply chain of a country when it was isolated from pork samples. In general, the detection frequency of MRSA from pigs at stunning to pork at retail decreases throughout the chain. These results indicate that LA-MRSA are transmitted along the chain and that the extent of MRSA transmission from pig to pork is limited in the course of slaughter. However, when drawing conclusions on potential MRSA transmission along the chain it has to be considered that although the reviewed investigations have been conducted within narrow regional and temporal parameters, the comparative compilation is not based on longitudinally collected data. Therefore, differences in the study designs concerning sampling plans and laboratory protocols limit the comparability of the results. The detailed analysis of the pig slaughter process leads to the assumption that especially process steps including superficial heat treatments like scalding and singeing can significantly reduce the burden of MRSA on the carcasses. However, recontamination with MRSA can occur via surface treating machinery, as a result of faecal contamination at evisceration or via increased human handling during meat processing. Therefore, transmission of MRSA from pig to pork can be minimized by optimizing processes with the potential towards carcass decontamination and avoiding recontamination primarily by effective cleaning and personal hygiene management.

LA-MRSA in connection with the pig sector has been reviewed before emphasizing variable features (11, 16, 20, 24, 26, 38). However, the present review is the first which pursues the approach of tracing LA-MRSA along the entire pork supply chain. The comparative compilation of MRSA prevalence and typing data separated by process step and region not only generates a structured view of the current state of research in this field but also provides first indications on potential MRSA transmission along the chain. Although the proposed descriptive approach is not able to establish any causal relationships, combined with detailed risk factor and process analysis the method is quite appropriate to develop the theoretical framework for further detailed transmission studies by determining LA-MRSA transmission routes and associated critical process steps.

14.2 Modeling the transmission of LA-MRSA along the pig slaughter chain

The comprehensive literature review of LA-MRSA in the pork supply chain supports the assumption that the slaughter process plays a decisive role for the extent of MRSA transmis-

sion from pig to pork. Therefore, a simulation model of the pig slaughter process was developed which describes the change in MRSA carcass prevalence during slaughter with special emphasis on identifying critical process steps for MRSA transmission. The model was used to quantify the impact of the initial MRSA herd prevalence of slaughter pigs on the outcome prevalence of the carcasses, to estimate the impact of cross contamination during slaughter, and to evaluate intervention strategies for minimizing the MRSA spread along the chain.

Mathematical models are frequently used in the course of risk assessment to trace the sources of microbial contamination in a food chain. They have proven their value as a tool to predict the effect of interventions in complex production processes and are used to assist decision processes in animal health policy for disease prevention and control (31). The pork production sector has been subject of model development before, describing the propagation of *Salmonella*, *Escherichia coli* and *Campylobacter* through the various stages of pork processing (1, 3, 15, 22, 36, 37). The underlying model frameworks differ significantly in statistical approach and complexity. First and foremost, the choice of approach should be appropriate for the scale of decision to be made. Quantitative microbial risk assessment models based on a farm to consumption approach are certainly a precise and specific evaluation of a process system. However, large effort and expertise in model construction and numerous high quality data are required to generate reliable inferences.

The transmission of MRSA along the pig slaughter chain has not been modeled yet. Due to the lack of any quantitative data on MRSA contamination levels on pigs and pig carcasses a simple model framework requiring less data is needed. Therefore, the modeling approach proposed in this thesis is based on prevalences as sole input variables. It was implemented on a modular chain of consecutive slaughter steps from scalding of the pigs to chilling of the final carcasses. As MRSA prevalence data were rare and just based on occasional sampling during process (4, 23, 29, 35), prevalence data of coagulase positive *Staphylococcus aureus* longitudinally sampled at several consecutive steps along the slaughter line were included and applied to MRSA (34). Differences between MRSA and its susceptible variant concerning the transmission and survival during the slaughter processes are not evident. These prevalences were assumed to exhibit a first order Markov property in the process chain where the MRSA status of an individual pig at a given processing step only depends on its status in the preceding production step (28). Thereby, the individual pig is able to change its state at each of the slaughter steps. Hence, the average value range of both the MRSA elimination and contamination rate of each of the slaughter processes were calculated and expected to follow a PERT distribution. A Monte Carlo simulation was set up for modeling the development of the MRSA contamination of pigs throughout slaughtering.

According to the model the MRSA herd prevalence has a low effect on the amount of positive pig carcasses at the end of the slaughter process. Consistently low outcome prevalences

between 0.15 and 1.15 % were calculated when varying the initial MRSA prevalence of the pigs at stunning between 5% and 95%. This result indicates that the pig slaughter process in general is able to reduce the MRSA detection frequency on pig carcasses to an acceptable level. In comparison, 11.7% MRSA positive samples of fresh pork portions were reported within the German national monitoring of zoonotic agents in 2009. The discrepancy in results might be due to MRSA cross contamination during meat preparation but might also indicate that improvement could be achieved. However, when interpreting the results it has to be considered that underlying data only represent two different Swiss abattoirs sampled in 2005. Any modernization in slaughter techniques could not be considered in the model. Although both abattoirs show a different course of positive pigs throughout the process which induces a wide variability in MRSA prevalence data the transferability of results to the German pig sector has to be compromised. However, if appropriate MRSA prevalence data from German abattoirs are available the representativeness of the model parameters can be improved.

As a next step, a sensitivity analysis was performed by stepwisely altering the values of both the elimination and contamination rates of each slaughter process between 0 and 1 and assessing the change in the outcome prevalence at the end of the slaughter. The proposed approach is an appropriate and simple method for identifying those process steps where a change in the contamination or elimination rate has a large effect on the outcome MRSA prevalence and specifying them as potential targets for process control and risk management. In general the alteration of contamination rates has a greater impact on the outcome prevalence than changing the elimination rates. The reduction of the elimination rate of scalding results in the highest increase of the outcome prevalence. The modification of the contamination rates is most effective if it is performed at final stages of the slaughter chain which might be partly influenced by the fact that the model is based on the Markov Chain principle. It can be concluded that scalding is a critical process step for MRSA transmission and that any cross contamination afterwards has to be avoided in order to obtain a low MRSA outcome prevalence.

Finally, the model was also used to quantify the impact of different deviances from optimal slaughter procedures by means of scenario analysis. In scenario 1, an insufficient scalding process was simulated. Cross contamination during dehairing/singeing and polishing was hypothesized within scenario 2. Scenario 3 which simulates the process of hot water spraying was based on scenario 2 with the addition of an increased decontamination during washing. All scenarios end with an increased MRSA prevalence ranging between 4.6 and 20.2% positive carcasses compared to a baseline value of 0.96%. Whereas the resulting higher MRSA prevalence after scalding could be reduced by subsequent process steps, simulating cross contamination during dehairing/singing and polishing leads to a significant increase of the MRSA outcome prevalence. This result confirms that cross contamination after singeing

is irreversible by subsequent slaughter steps. Simulating the application of decontamination technologies leads to a slight reduction of previous recontamination. This result was in line with previous investigations which reported spraying with hot water to yield only limited reduction of bacterial counts (44).

Mathematical modeling can only provide an approximation of actual process flows and the accuracy of its predictions is directly dependant from the quality and quantity of data underpinning it. The proposed framework differs from that of previous published pig slaughter process models (1, 3, 15, 22, 36, 37) as it is purely based on probabilistic considerations deduced from measured prevalence data. The inclusion of further assumptions in the form of expert opinion was waived thus enhancing the validity of results. As a consequence, the proposed approach includes a rough simplification of the rather complex pig slaughter system. As outcome, the model is able to quantify the change of MRSA prevalence during slaughter but cannot forecast the impact of single slaughter processes on the actual number of MRSA on carcasses. However, the level of detail is sufficient to identify critical process steps for MRSA cross-contamination and predict the effect of interventions on the outcome prevalence. As the model framework is rather non specific it can also be applied to other process chains and pathogens as long as prevalence data are available.

14.3 MRSA in the turkey meat supply chain

In 2010 the German national monitoring program for zoonotic agents included the evaluation of MRSA in the turkey meat production chain. Thereby, a significant increase in the MRSA prevalence after slaughter is revealed which is in contrast to the declining MRSA detection rate in the progressive course of pork production (9). In addition, an increased variability of CC398 associated *spa* types in meat samples compared to dust at farm or carcasses after slaughter was disclosed. Both results might lead to the conclusion that cross contamination of MRSA between the birds within a flock and between different flocks occurs during slaughter and therefore, the turkey slaughter process itself significantly contributes to the distribution of MRSA from stable to table. Regarding the transmission of *Salmonella* and *Campylobacter* during poultry slaughter these interrelations have been already shown (32, 40). Although the turkey meat supply chain was sampled within the relative short period of one year the monitoring has not been conducted in a longitudinal design. Therefore, a new approach is proposed for analyzing a cross sectional MRSA data set from different stages of the food chain with the intention to draw conclusions on potential farm to fork transmission. For this purpose, chi squared statistics was combined with the calculation of a similarity index to compare the distributions of specific characteristics of MRSA, the *spa* types, SCC*mec* types and antimicrobial resistance profiles, between the samples from turkeys at farm, car-

casses after slaughter and meat at retail. The degree of similarity is interpreted as reflecting MRSA transmission along the chain.

The chi-square test of homogeneity was used to determine whether *spa* types and antibiotic resistance profiles are distributed homogeneously within the MRSA samples from different steps of the turkey meat chain. As the wide variability in typing data would necessitate a higher number of samples in order to obtain adequate expected cell counts, the *spa* types were aggregated in 4 different categories corresponding to the frequency of occurrence. *Spa* types t011 and t034 dominating at each process step, built their own group whereas rare *spa* types of CC398 and all non CC398 strains were summarized separately. Although grouping of *spa* types not only diminishes the variability in data but also reduces the level of detail of the analysis, it was an acceptable compromise to achieve reliable results by chi-square statistics. The multidimensional data set of antibiotic resistance profiles was restructured using cluster analysis techniques. As the ordinal MIC values which are generated by two-fold dilutions in substance concentration are difficult to describe by cluster analysis a binary data set was generated by categorizing the MIC values for each isolate into resistant or susceptible according to the ECOFFs. The antimicrobial resistance profiles were then grouped by hierarchical cluster analysis using Ward's minimum variance and squared Euclidean distance. Pseudo-F (10) and Pseudo-T (13) statistics determined three different clusters with clear separation as no resistance phenotype simultaneously appeared in several clusters. This procedure has been shown to best separate binary antimicrobial resistance data before (7, 46). The distributions of SCC*mec* types in the different matrices were compared using Fisher's exact test as 33.3% of the cells of the contingency table had an expected value below 5 and grouping of the isolates was not sensible.

Chi squared statistics determines that the distribution of the groups of *spa* types, SCC*mec* types and the three clusters of antimicrobial resistance types did not significantly differ in the MRSA samples from turkey farms, carcasses after slaughter and meat at retail. Therefore, on the basis of the used data set it cannot be rejected that the MRSA isolates from different production steps within the turkey meat supply chain originate from the same population of strains. This result might rather support the hypothesis of farm to fork transmission of the same pool of MRSA strains than development of separate MRSA populations at each step of the chain.

As a second step, the similarity of the distribution of *spa* types, SCC*mec* types and antimicrobial resistance profiles within the MRSA samples from turkeys at farm, carcasses after slaughter and meat at retail were compared pair wise using the Czekanowski index. This index, which is also referred to as proportional similarity index (PSI), is an objective and simple method of quantifying the area of intersection between two frequency distributions. The values for similarity range from 1 for identical frequency distributions of the variable of inter-

est to zero for missing similarity. There is a wide variety of similarity indices which are notably used as standard analytical tools in community ecology (25). The Czekanowski index has also been applied to subtyping data in the course of source attribution studies (19, 30, 33). A comparison of the most common indices has shown that the Czekanowski approach most precisely reflects similarity for any underlying distribution (6). The index is intuitively and mathematically meaningful even in the case of empty cells in one or both of the distributions being compared (33). In addition, the index is independent from sample size and therefore any effects of differing sample sizes is excluded (25). As the size of the samples is rather small, a realization of the Czekanowski index may deviate from its true value. Thus, the index was bootstrapped (14). With this method, the three basic samples are treated as the population. A Monte Carlo algorithm was used for randomly sampling the data with replacement and generating a large number of bootstrap-samples of equal size as the original data sets. Each of these bootstrap-samples randomly departs from the original sample. Then the Czekanowski index was calculated from these resamples obtaining a probability density distribution from which we derived the mean and its 95% confidence interval.

Consistently high Czekanowski index values (0.79 – 0.86) could be calculated for the distribution of *spa* types and *SCCmec* types between the processing steps, indicating high similarity. The equivalent comparison of the distribution of antimicrobial resistance phenotypes observed medium index values (0.42 – 0.56) which might be due to the higher diversity of this characteristic in the sample set. This result suggests MRSA transmission along the chain. Higher Czekanowski index values were received by comparing the adjacent process steps primary production and slaughter as well as slaughter and meat in contrast to primary production and meat. This effect might reflect an increase in the variability of MRSA strains along the supply chain. Cross contamination of flock specific strains, the introduction of external strains into the process chain via human or environmental contamination or spontaneous mutations in the strains might explain the increasing number of different MRSA strains along the chain. A detailed process analysis confirms the suspicion that the turkey slaughter process contributes decisively to MRSA transmission from animal to meat. Turkey slaughtering is a very fast and highly automated process which does not include any step with the potential of carcass decontamination which contrasts with the pig slaughter process. Although scalding takes place the birds are only exposed to water temperature of 50 and 65°C for 60 to 210 sec (27), insufficient process parameters to reduce superficial MRSA counts. High throughput rates induce bacterial contamination of the treatment water leading to cross contamination (21). After scalding, the birds go through the plucking machine which has also been identified as a critical process step for microbial cross contamination (5, 27).

In order to validate if the proposed statistical method is in general able to detect existing differences in the sample sets the distribution of *spa* types, *SCCmec* types and antimicrobial

resistance profiles was also compared to a set of MRSA isolated from wild boar meat as an example of separated MRSA population. Thereby considerably lower Czekanowski index values were obtained with regard to *spa* types and antimicrobial resistance profiles than within the turkey chain. However, concerning SCC*mec* types, high index values were observed both between the samples of the turkey meat chain and in comparison to the control group indicating a low discriminatory power of SCC*mec* typing which might also be biased by the amount of not typeable SCC*mec* cassettes. These strains were considered to be homogeneous which might lead to an overestimation of similarity.

In the present study a similarity index was applied for the first time to a set of cross sectional MRSA data with the intention to prove transmission along a process chain. Based on the distribution of *spa* types and antimicrobial resistance types the proposed method appeared appropriate to draw conclusions on farm to fork transmission.

14.4 Tracing MRSA transmission along the veal production chain

The former proposed statistical approach was in part also applied to a farm to fork MRSA data set sampled from the key steps of the entire German veal production chain and thus supplements the methodological concept of a comprehensive representative investigation of the prevalence and strain diversity of MRSA in different cattle food chains in Germany. The MRSA data were generated in the course of the German monitoring program for zoonotic agents between 2009 and 2012 covering veal herds (dust from the stables), veal calves at slaughter (nasal swabs), carcasses of veal calves (surface cuts) as well as veal at retail. This sample set was analyzed pair wise using the Czekanowski index in order to estimate the degree of similarity of MRSA between the process steps on the basis of the frequency distribution of the different *spa* types within each sample category. Therefore, the *spa* types were categorized in the groups “t011”, “t034”, “other CC398” and “non CC398”, a classification which has already been proven as adequate within the former turkey study. Approximate confidence intervals of the Czekanowski index were calculated using the bootstrap method. This analysis revealed consistently high degrees of similarity (0.78 – 0.89) for all sample pairs. The comparison of MRSA from subsequent process steps within one sampling year results in the highest Czekanowski index values. These results suggest that MRSA are readily transmitted to the carcass during slaughter and also further down the food chain during processing. MRSA from meat distinguish most from the other sample categories. As 8.3% of these MRSA isolates could not be assigned to CC398 human contamination during processing has to be assumed. Comparing the different sampling years of 2009 and 2012 lower Czekanowski index values were calculated suggesting a gradual change in the distribution of *spa* types in the veal population with the years.

In conclusion, the hypothesis that livestock associated MRSA are transferred along the pork, poultry and beef production chain from animals at farm to meat on consumers' table can be confirmed by the methodological concepts developed in the present thesis.

The proposed simulation model extends the spectrum of methods for bacterial transmission assessment. As the framework has comparatively low data requirements and is not specific to process chains or pathogens it offers a broad field of application. With regard to the poultry meat production chain the model framework could help to develop concrete improvement suggestions to optimize the slaughter process with the intention to reduce the massive MRSA transmission down this chain. However, appropriate MRSA prevalence data would first have to be collected.

The combination of chi squared statistics and the Czekanowski index has demonstrated its value to assess MRSA transmission along the food production chain. The method is appropriate to expand the statistical evaluation routines of the German national monitoring program for zoonotic agents as it allows both, the continuous assessment of bacterial transmission dynamics from farm to fork as well as the early recognition of changes in the distribution of individual genetic lineages over time if data sets from different sampling years are compared. Thereby, the proposed approach can be adapted to various pathogens and food chains.

15 References General Discussion

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16 List of publications

1. Lassok, B.*, and B. A. Tenhagen. 2013. From pig to pork: Methicillin-resistant *staphylococcus aureus* in the pork production chain. Journal of Food Protection. 76:1095-1108.
2. Vossenkuhl, B., H. Sharp, J. Brandt, A. Fetsch, A. Käsbohrer, and B.-A. Tenhagen. 2014. Modeling the transmission of livestock associated methicillin-resistant *Staphylococcus aureus* along the pig slaughter line. Food Control. 39:17-24.
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* Lassok is the maiden name of Birgit Vossenkuhl

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18 Author's declaration

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere weiterhin, dass alle anderen Werken wörtlich oder inhaltlich entnommenen Stellen als solche gekennzeichnet wurden.

Die Arbeit wurde bisher keiner anderen Prüfungsbehörde vorgelegt.

Berlin, den 23.5.2015

(Birgit Vossenkuhl)