Aus dem

Bundesinstitut für Risikobewertung

und dem Fachbereich Veterinärmedizin

der Freien Universität Berlin

Methicillin resistant staphylococci on German dairy farms

Aspects of distribution, transmission, and control on farm level

Inaugural-Dissertation

zur Erlangung des Grades eines

Doktors der Veterinärmedizin

an der Freien Universität Berlin

vorgelegt von

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Tierarzt aus Göttingen

Berlin 2021

Journal-Nr: 4287

Gedruckt mit Genehmigung

des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

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Deskriptoren (nach CAB-Thesaurus):

dairy cows, dairy farms, dairy herds, staphylococcus, methicillin resistant staphylococcus aureus, disease prevalence, disease transmission, milk, methicillin, antibiotics, drug resistance, environment, epidemiology, Germany

Tag der Promotion: 03.09.2021

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List of Abbreviations

AMR	Antimicrobial resistance
BTM	Bulk tank milk
CA	Community associated
CC	Clonal complex
CI	Confidence interval
CM	Clinical mastitis
EFSA	European Food Safety Authority
HA	Healthcare associated
IEC	Immune evasion cluster
l	Litre
LA	Livestock-associated
MALDI-TOF	Matrix assisted laser desorption/ionization- time of flight
mg	Milligram
ml	Millilitre
MLST	Multi locus sequence typing
MR-NAS	Methicillin resistant non-*aureus* staphylococci
MRS	Methicillin resistant staphylococci
MRSA	Methicillin resistant *Staphylococcus aureus*
NAS	Non-*aureus* staphylococci
OR	Odds ratio
PCR	Polymerase chain reaction
PFGE	Puls field gel electrophoresis
pvl	Panton Valentine Leukocidine
QMS	Quarter milk sample
S.	*Staphylococcus*
SCC	Staphylococcal cassette chromosome
spa	Staphylococcal protein A
ST	Sequence type
t	Staphylococcal protein A type
tsst	Toxic shock syndrome toxin
VGP	Viertelgemelksproben

1 Introduction

1.1 Mastitis in dairy cows

Mastitis is the most prevalent infectious disease in dairy cows worldwide (Ruegg, 2017a). In Germany, approximately 13% of all cow losses in 2019 were due to mastitis (BRS 2020). Mastitis negatively impacts animal welfare, milk quality and financial profit on dairy farms (Steeneveld et al. 2011; Gonçalves et al. 2018). Inflammation is mostly caused by bacteria and their toxins invading the udder. Unusual infectious agents are viruses, fungi and algae (Wellenberg et al. 2002; Blowey and Edmondson 2010). In some cases, physical trauma and chemical irritants may cause mastitis (NMC 2017). Common signs of clinical mastitis are clots in milk as well as heat, hardness and swelling of the udder (Blowey and Edmondson 2010; NMC 2017). Subclinical mastitis is usually detected by bacterial culture and somatic cell count measurements in milk. Since most somatic cells in milk are immune cells, a high somatic cell count indicates an inflammatory process in the cows' udder. The cell count cut-off values for mastitis detection in milk of dairy cows differ between studies. Cut-off values between 70,000 cells/ml and 250,000 cells/ml have been suggested (Laevens et al. 1997; Djabri et al. 2002; De Vliegher et al. 2012). The most common mastitis causing pathogens in dairy cows are staphylococci, streptococci and coliforms (Blowey and Edmondson 2010; Ruegg 2017a). Based on the primary reservoir of infection, mastitis causing pathogens are divided in environmental and contagious pathogens. Environmental species usually persist in the barn environment (e.g. bedding materials) and contagious pathogens are associated with the cows' udder. This classification is important since prevention and control strategies for contagious and environmental mastitis causing pathogens are different. To date, environmental mastitis causing pathogens like *Streptococcus uberis* and *Escherichia coli* are the most frequently detected bacteria from clinical mastitis milk samples in most countries with modern dairy industries (Oliveira et al. 2013; Gao et al. 2017; Ruegg 2018). In the past decades, the overall burden of contagious mastitis causing pathogens like *Staphylococcus (S.) aureus* and *Streptococcus agalactiae* has been reduced by the implementation of antibiotic therapy, milking time hygiene and culling of infected animals (Blowey and Edmondson 2010; NMC 2017; Ruegg 2017a).

1.2 *Staphylococcus aureus*

S. aureus is characterized as a gram positive, facultative anaerobic, immobile, catalase and coagulase positive coccoid bacterium, that forms grape-like clusters. *S. aureus* is ubiquitous and serves as an opportunistic pathogen. In humans and animals, *S. aureus* is mainly associated with skin and mucous membranes. In veterinary medicine, *S. aureus* causes

diseases in different animal species, however bovine mastitis is economically the most important one (Peton and Le Loir 2014).

1.2.1 *S. aureus* as a mastitis pathogen

Since the 1970's, *S. aureus* infections in dairy herds were reduced by the implementation of control programs that focused on dry cow therapy, milking time hygiene and culling of chronically infected animals (Neave et al. 1969; NMC 2017). Nevertheless, *S. aureus* is still among the most frequently detected mastitis causing pathogens in many countries (Østerås 2018; Ruegg 2018). *S. aureus* is considered a contagious pathogen in dairy herds that spreads from cow to cow and from quarter to quarter especially during the milking process. Cows that carry *S. aureus* in the mammary gland are the main reservoir of infection within herds (Keefe 2012). New infections mostly occur during lactation and rarely in the dry period. *S. aureus* mostly causes subclinical infections of long duration (Sears and McCarthy 2003; Barkema et al. 2006). Therefore, *S. aureus* affected herds suffer from elevated bulk tank milk (BTM) somatic cell counts and significant milk losses. In individual cases, peracute gangrenous staphylococcal mastitis caused by *S. aureus* may occur (Blowey and Edmondson 2010). The pathogenesis of *S. aureus* in the udder is complex and not fully understood (Naushad et al. 2020). After invading the udder through the teat canal, *S. aureus* has the ability to survive inside mammary epithelial cells and immune cells (Barkema et al. 2006, Kerro Dego et al. 2002). Moreover, *S. aureus* forms deep-seated pockets of infection and (micro-) abscesses (Zecconi and Scali 2013; Magro et al. 2017). Therefore, infections may persist over a long period and antimicrobial therapy is often not successful.

1.2.2 *S. aureus* and human health

In humans, *S. aureus* causes mild skin infections but also more severe diseases like implant infections, pneumonia, endocarditis and blood stream infections (David and Daum 2017).
S. aureus strains from bovine mastitis are usually different from human associated isolates indicating a low risk for zoonotic infections on dairy farms (Holmes and Zadoks 2011; Zadoks et al. 2011; Peton and Le Loir 2014). The risk for *S. aureus* transmission into the general population via dairy products seems to be low since milk is usually heat treated before marketing and consumption. However, the consumption of *S. aureus* contaminated raw milk and raw milk products may pose a risk for human health. In addition, some *S. aureus* strains produce toxins during growth in milk and raw milk products that are not cooled adequately.

After ingestion, these toxins can cause food posing symptoms like vomiting and abdominal cramps (Fetsch and Johler 2018).

1.2.3 *S. aureus* molecular typing methods

Different methods have been used for molecular characterization of *S. aureus* strains. Pulsed-field gel electrophoresis (PFGE) has been used for separation of long DNA molecules to detect short-term genetic variation (Golding et al. 2015). Typing of the staphylococcal protein A (*spa*-typing) and multi locus sequence typing (MLST) are focused on slowly accumulating genetic variation. For *S. aureus* MLST typing, seven housekeeping genes are used to determine a specific sequence type (ST) (Saunders and Holmes 2007). If sequence types differ by only one allel, they belong to the same clonal complex (CC). The *spa*-type is determined by sequencing of a 24-base-pair repeat within the staphylococcal protein A sequence (Fasihi et al. 2017). While PFGE can be used for short term outbreak analysis, *spa*-typing and MLST typing are used for large populations and global epidemiological investigations (Holmes and Zadoks 2011). To study genetic relationships in depth, as well as resistance and virulence mechanisms, whole genome sequencing (WGS) is the preferred molecular typing method (Naushad et al. 2020).

In Switzerland, a new PCR approach for *S. aureus* detection on herd level was developed, that becomes more and more adapted worldwide (Graber et al. 2007; Boss et al. 2011). *S. aureus* isolates are grouped in certain genotypes (especially genotype B and genotype C) that allow a prediction of contagiousness and pathogenicity of the *S. aureus* strains in dairy herds (Leuenberger et al. 2019). Subsequent strain-specific sanitation procedures have been recommended (Graber 2020).

1.3 Non-*aureus* staphylococci

Non-*aureus* staphylococci are a diverse bacterial group of currently 55 different species (Parte et al. 2020). In contrast to *S. aureus*, most NAS species do not produce the coagulase enzyme. Therefore, the tube coagulase test was traditionally used for *S. aureus* identification in clinical practice and NAS were formerly referred to as ‘coagulase negative staphylococci’. NAS carry fewer virulence-associated genes than *S. aureus* and are considered pathogens of minor importance for udder health (Åvall-Jääskeläinen et al. 2018). In milk samples, NAS were more frequently detected in primiparous cows, in clinically normal quarters and in herds with low BTM somatic cell counts (Sampimon et al. 2009; Schukken et al. 2009; Condas et al. 2017). Consequently, the proportion of NAS among mastitis causing pathogens is higher on well-

managed farms with overall good udder health. The implementation of NAS species identification raised concerns about the existing classification of NAS as a single group of mastitis causing pathogens. Recent studies provide evidence that some NAS species should be regarded as environmental opportunistic pathogens, while others seem to be udder adapted and more pathogenic species (De Visscher et al. 2014; Wuytack et al. 2020b).

In human medicine, NAS are considered opportunistic pathogens. The majority of the healthy human population carries NAS in the nasal cavities (Becker et al. 2006). Severe NAS infections are mainly associated with immunocompromised patients and surgical procedures (Becker et al. 2020). Due to the ongoing medical progress and the subsequent increasing number of immunocompromised patients, the proportion of NAS infections in human patients is increasing (Becker et al. 2020).

1.4 Methicillin resistant staphylococci

1.4.1 Antimicrobial resistance

Antimicrobial resistance (AMR) is a natural process in which microorganisms acquire the ability to overcome a pharmaceutical treatment that was designed to restrict or kill them. Leading public health institutions declared that AMR is one of the most important threats to human and animal health worldwide (O'Neill 2016). AMR causes treatment failure, a higher mortality and increased overall costs for treatment of infections caused by resistant pathogens. The use of antibiotics has been considered the major cause of AMR since resistant bacteria have a competitive advantage within a treated population (Chantziaras et al. 2014). In dairy herds, mastitis is the most common indication for antimicrobial therapy (Pol and Ruegg 2007, Hommerich et al. 2019). Especially the use of high-dosage long-acting antimicrobials in dry cow formulations has been considered a driver of AMR in dairy cows (Saini et al. 2012b). However, blanket dry cow therapy has been used for decades and several studies provide evidence that resistance levels are not increasing among mastitis causing pathogens from dairy cows (Eskrine et al. 2004; Oliver and Murinda 2012; Ruegg 2017b).

1.4.2 Methicillin resistant *Staphylococcus aureus*

The β-lactam antimicrobial ‘Penicillin’, was the first antimicrobial drug in human medicine (Aminov 2017). Penicillin restricts the bacterial cell wall synthesis of staphylococci, resulting in death of the bacteria. The first mechanism by which staphylococci became resistant to penicillin was by the production of β-lactamase enzymes in the 1940’s (Kirby 1944). β-

lactamase enzymes hydrolyze and disrupt the internal structure of the penicillin, rendering the drug ineffective. In the 1960's, methicillin, the first semi-synthetic penicillin, was introduced in hospitals. Methicillin and other semisynthetic penicillins are resistant to the action of penicillinase enzymes. To date, methicillin is not used anymore while oxacillin and cloxacillin are common semisynthetic penicillins in clinical practice. Shortly after the introduction, the first methicillin resistant *S. aureus* (MRSA) were detected (Dowling 1961; Jevons 1961). In this case, broad-spectrum β-lactam resistance was not mediated by β-lactamase but rather by a modified penicillin binding protein 2a (PBP2a). The PBP2a is usually encoded by the *mecA* or *mecC* gene, which is located on a gene cassette called 'staphylococcal cassette chromosome *mec'* (SCC*mec*). The SCC*mec* is a mobile genetic element that may be transferred between staphylococcal species (Hanssen and Ericson Sollid 2006; Miragaia 2018). Based on the genotypic diversity, groups of SCC*mec* types (I-XIII) were determined, which are important epidemiological markers in MRSA research. The SCC*mec* gene cassette often harbors additional resistance genes for example against aminoglycosides, macrolides and fluoroquinolones (Oliveira et al. 2000; Ikawaty et al. 2009). Therefore, MRSA are mostly considered 'multi-drug resistant' pathogens.

From an epidemiological standpoint, MRSA have been classified as healthcare-associated (HA-), community-associated (CA-) and livestock-associated (LA-) MRSA strains (Mehraj et al. 2016). HA-MRSA are responsible for nosocomial infections worldwide and usually harbor SCC*mec* types I, II or III. Since the 1990s, CA-MRSA were increasingly detected in humans with little or no contact to hospitals and other healthcare settings. These MRSA strains mostly carry SCC*mec* elements IV and V as well as the *pvl* gene, which encodes for the cytotoxin 'Panton-Valentin leucocidin'. The third category of MRSA is associated with livestock (LA-MRSA). The predominant SCC*mec* types in LA-MRSA are IVa and V and in Europe they usually belong to the multi locus ST398. LA-MRSA ST398 were detected in the Netherlands for the first time (Armand-Lefevre et al. 2005). The most frequently affected animals are pigs with LA-MRSA detection rates of up to 89% in Europe (Porrero et al. 2012; Abreu et al. 2019). LA-MRSA ST398 were also detected in other animal species including companion animals, horses, veal calves, chicken, turkeys, mink and rodents (Nemati et al. 2008; Graveland et al. 2010; Sieber et al. 2011; Vincze et al. 2014, Hansen et al. 2017). In humans, LA-MRSA are mainly transient opportunistic pathogens affecting people who work in close contact with livestock (Fluit 2012; Cuny et al. 2015). Individual cases of life-threatening infections caused by LA-MRSA ST398 were described, which underlines the possible zoonotic risk (Goerge et al. 2017). In Germany, approximately 4% of clinical human MRSA isolates belonged to ST398, indicating a minor impact on human health (Layer et al. 2019). However, in German regions with high pig density (e.g. Münsterland) and in Scandinavian countries, where the number of

HA-MRSA is traditionally low, the proportion of LA-MRSA ST398 among human isolates was up to 35% (DANMAP 2016; Van Alen et al. 2017). The transfer of LA-MRSA to humans via raw milk and raw milk products might be possible. Calves carried LA-MRSA in their nasal cavities after feeding of MRSA contaminated milk (Spohr et al. 2011).

1.4.3 Methicillin resistant non-*aureus* staphylococci

In methicillin resistant non-*aureus* staphylococci (MR-NAS), the diversity of *mecA* homologs and SCC*mec* elements is larger compared to MRSA, indicating a key role of NAS in the evolution of β-lactam resistance (Miragaia 2018). The *mecA* gene as well as the different SCC*mec* types probably evolved in NAS from the *S. sciuri* group and were subsequently transferred to other staphylococci (Rolo et al. 2017). Various studies showed that SCC*mec* elements can be exchanged between staphylococcal species in vitro (Hanssen and Ericson Sollid 2006; Morikawa et al. 2012; Ray et al. 2016). Therefore, NAS as pathogens of minor importance for human and animal health, may serve as a reservoir of resistance genes for the major pathogen *S. aureus*. To what extend and by which mechanisms resistance genes are exchanged between staphylococcal species in vivo is largely unknown.

The most common MR-NAS species from milk of dairy cows are summarized in Table 1. MR-*S. sciuri* and MR-*S. epidermidis* were the most frequently detected species in previous studies. Among NAS pathogens from humans, methicillin resistance is increasing worldwide (Malhas et al. 2015; Chen et al. 2018). In previous studies, most clinical *S. epidermidis* and *S. haemolyticus* isolates exhibited broad-spectrum β-lactam resistance (Barros et al. 2012; Mendes et al. 2012; Deplano et al. 2016). It was suggested that the occurrence of MR-NAS in animals may contribute to the resistance situation in humans, since staphylococci and resistance genes can be exchanged between humans and animals (Becker et al. 2020).

Table 1. Methicillin resistant non-*aureus* Staphylococci in milk samples from dairy cows

Author/Year	Country	Number of samples and sample type	MR-NAS species (n)
Frey et al., 2013	AT	417 NAS from 370 milk samples	*S. sciuri* (37) > *S. fleuretti* (11) > *S. epidermidis* (6) > *S. haemolyticus* (1), *S. xylosus* (1)
Huber et al., 2011	AT	100 BTM samples	*S. fleurettii* (37) > *S. sciuri* (11),>*S. cohnii* (1), *S. haemolyticus* (1)
Chehabi et al., 2019	DK	49 NAS from CM	*S. chromogenes* (1)
Cicconi-Hogan et al., 2014	USA	288 BTM samples	*S. sciuri* (5) > *S. saprophyticus* (3) > *S. chromogenes* (2) > *S. agentis* (1)
De Jong et al., 2018	EU	165 NAS from CM	*S. epidermidis* (3) > *S. sciuri* (1), *S. saprophyticus* (1), *S. huyicus* (1)
Fisher and Paterson, 2020	UK	363 BTM samples	*S. sciuri* (6) > *S. epidermidis* (4) > *S. saprophyticus* (3) > *S. fleurettii* (1), *S. lentus* (1)
Fessler et al., 2010	DE	121 NAS from mastitis milk samples	*S. epidermidis* (8) > *S. haemolyticus* (5) > *S. saprophyticus* (1), *S. capitis* (1)
Nobrega et al., 2018b	CA	405 NAS from mastitis milk samples	*S. epidermidis* (4)
Sampimon et al., 2011	NL	170 NAS from milk samples	*S. epidermidis* (7) > *S. chromogenes* (6) > *S. fleurettii* (5) > *S. sciuri* (3) > *S. warnii* (1), *S. succinus* (1), *S. equorum* (1)
Seixas et al., 2014	PT	204 Staphylococci from mastitis milk samples	*S. epidermidis* (16) > *S. chromogenes* (1), *S. simulans* (1), *S. haemolyticus* (1)
Gindonis et al., 2013	FI	434 mastitis milk samples	*S. epidermids* (18) > *S. fleuretti* (1)
Kim et al., 2019	KP	311 NAS from mastitis milk samles	*S. epidermidis* (18) > *S. sciuri* (1), *S. hominis* (1), *S. equorum* (1)

Table 1. Continued: Methicillin resistant non-*aureus* Staphylococci in milk samples from dairy cows

Author/Year	Country	Number of samples and sample type	MR-NAS species (n)
Fernandes Dos Santos et al. 2016	BR	91 NAS from milk samples	*S. epidermidis* (10)
Qu et al., 2018	CN	112 NAS from CM	*S. chromogenes* (26) > *S. sciuri* (14) > *S. epidermidis* (8) > *S. simulans* (5), *S. equorum* (5), *S. hominis* (5) > *S. haemolyticus* (4), *S. argenteus* (4)
Vanderhaeghen et al., 2013	BE	100 nasal swabs from dairy cows	*S. sciurii* (10) > *S. epidermidis* (2) > *S. fleurettii* (1)
Khazandi et al., 2018	AU	37 NAS from 320 milk samples	*S. sciuri* (5) > *S. succinus* (2) > *S. haemolyticus* (1), *S. fleurrettii* (1)
Taponen et al., 2016	FI	400 NAS from mastitis milk samples	*S. epidermidis* (20) > *S. sciuri* (1)
Moon et al., 2007	KP	763 NAS from 3047 mastitis milk samples	S. *saprophyticus* (5) > *S. epidermidis* (4), *S. simulans* (4) > *S. sciuri* (3) > S. xylosus (1), intermedius (1), *S. hominis* (1)
Mello et al., 2017	BR	181 Staphylococci from mastitis milk samples	*S. epidermidis* (8)
Wuytack et al. 2020a	BE	59 NAS from mastitis milk samples	*S. haemolyticus* (9) > *S. epidermidids* (4) > *S. equorum* (3) > *S. xylosus* (2) > *S. lentus* (1), *S. sciuri* (1), *S. simulans* (1), *S. capitis* (1), *S. succinus* (1)

BTM: Bulk tank milk
CM: Clinical mastitis
NAS: Non-*aureus* staphylococci
MR-NAS: Methicillin resistant non-*aureus* staphylococci
Countries: AT- Austria, DK- Denmark, USA- United States of America, EU- Europe, UK- United Kingdom, DE- Germany, CA- Canada, NL- Netherlands, PT- Portugal, FI- Finland, KP- Korea, BR- Brazil, CN-China, BE- Belgium, AU- Australia

2 Outlines and objectives

Methicillin resistant staphylococci (MRS) can cause mastitis in dairy cows (Locatelli et al. 2017). Infections caused by MRS are a problem in veterinary medicine because of limited treatment options. Some MRS spread between different animal species and may concern human health (Cuny et al. 2015). Consequently, possible human and animal health hazards provide a rational for research on MRS in dairy herds.

To reduce the emergence and spread of MRS on dairy farms, it is crucial to identify new MRS reservoirs and possible routes of transmission. This dissertation therefore aimed to investigate aspects of MRS distribution, transmission, and control on dairy farms. In detail, the objectives were 1) to analyze the occurrence and transmission of MRS on dairy farms, 2) to determine the impact of MRS on udder health of dairy cows and 3) to develop MRS monitoring, prevention, and control recommendations for dairy herds. To address these objectives, the following research steps were conducted:

I. Literature review on MRSA in dairy herds
   A comprehensive review was performed to summarize previous research on methicillin resistant *Staphylococcus aureus* (MRSA) in dairy herds worldwide. A special focus was set on potential risk factors for the occurrence of MRSA in dairy herds.

II. Field study on 20 German dairy farms
   In the field study, samples from different age groups of cattle, humans, and the environment of 20 dairy farms were collected for bacterial culture. Farms were selected based on previous MRSA findings. A questionnaire, observations during the milking process and herd management software were used for analysis of milking time hygiene, biosecurity measures and general farm management data.

III. Bacterial culture and molecular characterization
   Presumptive MRS were cultured using a two-step selective enrichment method. Species were identified using MALDI-TOF mass spectrometry. The *mecA* and *mecC* gene were detected using established PCR protocols. MRSA *spa*-typing and SCC*mec*-typing were performed for epidemiological analysis. Thirty-three MRSA isolates were selected for whole genome sequencing to analyze genetic relationships in depth as well as virulence and resistance genes

3 Publications

3.1 Publication 1

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Risk Factors for the Occurrence of Methicillin-Resistant *Staphylococcus aureus* in Dairy Herds – An Update

https://doi.org/10.1089/fpd.2019.2638

Foodborne Pathogens and Disease

October 2020; 17(10); 585-596.

FOODBORNE PATHOGENS AND DISEASE
Volume 17, Number 10, 2020
Mary Ann Liebert, Inc.
DOI: 10.1089/fpd.2019.2638

Review Article

Risk Factors for the Occurrence of Methicillin-Resistant *Staphylococcus aureus* in Dairy Herds: An Update

Arne Schnitt and Bernd-Alois Tenhagen

Abstract

In dairy cows, *Staphylococcus aureus* is a major mastitis pathogen and methicillin-resistant *S. aureus* (MRSA) has been reported from dairy farms around the world. The risk of foodborne zoonotic infections with bovine MRSA strains seems to be low since MRSA prevalence is low in dairy herds and milk is commonly heat treated before consumption. However, bovine mastitis caused by MRSA is an important issue in veterinary medicine since treatment options with non-β-lactam antibiotics are limited. For the development of effective MRSA prevention strategies, it is necessary to know which factors increase the risk for MRSA transmission into and within dairy herds. Therefore, the aim of this review is to summarize the risk factors for the occurrence of MRSA in dairy herds and to identify the respective knowledge gaps. MRSA was more frequently detected in conventional dairy farms than in organic farms and in larger farms than in smaller farms. Dairy farms housing pigs along with cattle are more frequently affected by MRSA. Moreover, humans carrying MRSA can probably infect dairy cows. Consequently, pigs and humans may introduce new MRSA strains into dairy herds. MRSA transmission within dairy herds was associated with improper milking hygiene procedures. Furthermore, methicillin-resistant coagulase-negative staphylococci (MR-CoNS) were repeatedly isolated from dairy farms. This is an important issue since MR-CoNS may transfer resistance genes to *S. aureus*. The role of antimicrobial exposure as a risk factor for the occurrence of MRSA within dairy herds needs to be further investigated.

Keywords: methicillin, staphylococcus, MRSA, dairy, milk

Introduction

Staphylococcus aureus is considered a contagious mastitis pathogen that enters the mammary gland through the teat canal. In most cases there is one predominant *S. aureus* strain that affects multiple cows and spreads from cow to cow within dairy herds (Zadoks *et al.*, 2000; Barkema *et al.*, 2006; Keefe, 2012). Thus, the primary risk period for *S. aureus* transmission is during the milking process. The usual routes of transmission are milkers' hands, udder cloths, and milking equipment such as teat liners.

The overall prevalence of mastitis pathogens is highly variable and differs between herds and regions. To date, the most common pathogens causing clinical mastitis seem to be environmental streptococci and coliform bacteria followed by *S. aureus* (Ruegg, 2018). In some studies, *S. aureus* is still the most prevalent pathogen isolated from mastitis milk samples (Østerås, 2018).

In *S. aureus*, methicillin resistance is mediated by a *mecA*- or *mecC*- gene. This gene is located on a mobile genetic element called ''staphylococcal cassette chromosome *mec*'' (SCC*mec*). The gene is responsible for the production of an altered penicillin-binding protein 2a (PBP2a). The PBP2a has a lower affinity for β-lactam antimicrobials than the normal PBP. Thus, *mecA-/mecC*-positive staphylococci are resistant to most β-lactam antibiotics (Holmes and Zadoks, 2011; Miragaia, 2018).

While cure rates for lactational *S. aureus* treatments are low, dry cow therapy (DCT) is typically more effective (Keefe, 2012). Most frequently recommended dry cow antibiotics for the treatment of methicillin-sensitive *S. aureus* (MSSA) infections contain β-lactams (Tenhagen *et al.*, 2006; Saini *et al.*, 2012a). Especially cloxacillin is extensively used on dairy farms and cure rates for dry cow treatment of *S. aureus* infections with cloxacillin were reported to range up to 98% (Makovec and Ruegg, 2003a; Tenhagen *et al.*, 2006;

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Saini *et al.*, 2012c). Although, there are no studies on antibiotic treatment outcomes for mastitis caused by methicillin-resistant *S. aureus* (MRSA), cloxacillin, and other β-lactams are probably ineffective. Consequently, culling might be the only chance to remove MRSA from dairy herds. In addition, MRSA in dairy cows is of human health concern since people working on dairy farms were shown to carry similar MRSA strains as their cows (Juhasz-Kaszanyitzky *et al.*, 2007; Hata *et al.*, 2010; Spohr *et al.*, 2011; Lim *et al.*, 2013; Locatelli *et al.*, 2017). In these studies, the direction of transmission remained unclear. MRSA transmission from cows to consumers of milk seems unlikely due to commonly practiced heat treatment. However, the consumption of raw milk is a possible source of infection (Al-Ashmawy *et al.*, 2016; Parisi *et al.*, 2016). This might be an issue since many dairy farmers and their families consume raw milk and the number of raw milk vending machines is increasing in Europe (www.milkmaps.com). Thus, MRSA in dairy herds represents a possible health hazard for both humans and cattle. The objective of this review is to summarize the risk factors for the occurrence and spread of MRSA in dairy herds and to identify the respective knowledge gaps.

Prevalence and Epidemiology of MRSA in Dairy Herds

The detection of *S. aureus* in dairy cows is demanding due to its intermittent shedding patterns in milk (Barkema *et al.*, 2006; Keefe, 2012). Comparison of MRSA prevalence studies is additionally challenging because of differences in types of samples, inoculum volumes, (pre-) enrichment, and detection methods.

MRSA prevalence (*mecA*/*mecC*) in bulk tank milk (BTM) has been previously reported to range from 0% to 20% (Table 1). A study from Sicily found a significantly higher MRSA prevalence of 43.8% in BTM from dairy farms (Antoci *et al.*, 2013). This high prevalence was presumably caused by the preselection of dairy farms that had been tested positive for MRSA in previous years. The average MRSA prevalence from all other BTM samples in Table 1 is ~2.9%. The majority of studies (76%) are from Europe. MRSA prevalence was significantly lower in BTM samples from the United States with ~0.3% (3/980) (Virgin *et al.*, 2009; Haran *et al.*, 2012; Cicconi-Hogan *et al.*, 2014). Compared with Europe, MRSA prevalence was also lower in pig herds from the United States (Sun *et al.*, 2015; Abreu *et al.*, 2019). As shown in Table 2, the MRSA prevalence of *S. aureus* mastitis isolates was reported to be between 0% and 49%. The average MRSA prevalence of all individual milk samples in Table 2 is ~4.5%. The MRSA prevalence within individual dairy herds is shown in Table 3. The highest within-herd prevalence of MRSA was 39.7% (31/78) in Japan, 44% (11/25) in Sweden, and 60% (n=33/55) in a herd from Italy (Hata, 2016; Locatelli *et al.*, 2017; Unnerstad *et al.*, 2018).

The overall MRSA prevalence in dairy herds is low, compared with other animal species, especially pigs. However, reports from Korea and Germany indicate that MRSA prevalence rates might be increasing over time. In Germany, the prevalence of MRSA-positive BTM samples increased from 4.1% in 2009 over 4.7% in 2010 to 9.7% in 2014 (Tenhagen *et al.*, 2014, 2018). The German studies included BTM samples from all over Germany. The studies were performed under similar conditions within the framework of a national monitoring program. In Korea, MRSA prevalence was up to 6% until 2003 and 13.9% in 2011–2012 (Kwon *et al.*, 2005; Moon *et al.*, 2007; Song *et al.*, 2016). The Korean studies tested mastitis milk samples from different regions in Korea and their comparability is therefore difficult to evaluate. The authors of the last study concluded that the prevalence of MRSA in mastitis milk has continuously increased in Korea (Song *et al.*, 2016). In conclusion, there is some evidence that MRSA prevalence might be increasing in some countries.

In Europe, livestock-associated MRSA (LA-MRSA) belonging to clonal complex 398 (CC398) are the predominant MRSA strains in dairy herds. They were repeatedly isolated from milk samples (Fessler *et al.*, 2010; Vanderhaeghen *et al.*, 2010; Kreausukon *et al.*, 2012; Paterson *et al.*, 2012; Tavakol *et al.*, 2012; Tenhagen *et al.*, 2014, 2018; Luini *et al.*,

Table 1. Methicillin-Resistant *Staphylococcus aureus* Prevalence in Bulk Tank Milk from Dairy Cows

References	*MRSA in BTM % (n=MRSA/n=samples)*	*Year(s) of collection*	*Country*
Antoci *et al.* (2013)	43.8 (21/48)	2010	Italy
Cicconi-Hogan *et al.* (2014)	0.03 (1/288)	2009–2011	United States
Cortimiglia *et al.* (2016)	3.8 (32/844)	2012–2013	Italy
Haran *et al.* (2012)	1.3 (2/150)	2009	United States
Kreausukon *et al.* (2012)	4.4 (28/635)	2009–2010	Germany
Locatelli *et al.* (2016)	4.0 (9/224)	2011	Italy
Obaidat *et al.* (2018)	20.0 (16/80)	2015–2016	Jordan
Papadopoulos *et al.* (2018)	10.0 (1/10)	2016	Greece
Parisi *et al.* (2016)	2.5 (12/486)	2012–2013	Italy
Paterson *et al.* (2012)	0.5 (7/1500)	2012	United Kingdom
Paterson *et al.* (2014)	2.4 (11/465)	2011–2012	United Kingdom
Ronco *et al.* (2018)	0.0 (0/94)	2016	Denmark
Tenhagen *et al.* (2014)	4.4 (28/635)	2009–2010	Germany
Tenhagen *et al.* (2018)	9.7 (36/372)	2014	Germany
Virgin *et al.* (2009)	0.0 (0/542)	2007	United States
Visciano *et al.* (2014)	0.0 (0/30)	—	Italy
Vyletělova *et al.* (2011)	2.8 (20/703)	—	Czech Republic, Slovakia

MRSA was defined as *mecA*/*mecC*-positive *S. aureus* strains.
BTM, bulk tank milk; MRSA, methicillin-resistant *S. aureus*.

TABLE 2. METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* PREVALENCE OF *STAPHYLOCOCCUS AUREUS* ISOLATES DETECTED IN INDIVIDUAL MILK SAMPLES FROM MORE THAN ONE FARM

References	*Total No. of milk samples (n)*	*% MRSA of* S. aureus *isolates (n = MRSA/n = S.* aureus*)*	*Year(s) of collection*	*Country*
Ahangari *et al.* (2017)	—	1.3 (1/75)	2014–2015	Iran
Aslantas and Demir (2016)	330	4.5 (5/112)	2008–2010	Turkey
Bao *et al.* (2016)	121	9.6 (5/52)	—	China
Bardiau *et al.* (2013)	—	4.4 (19/430)	2005–2008	Belgium
Bengtsson *et al.* (2009)	987	0 (0/211)	2002–2003	Sweden
Bervoets (2009)	—	0 (0/550)	—	Canada
Dan *et al.* (2018)	186	16.3 (16/98)	—	China
da Costa Krewer *et al.* (2015)	2064	0 (0/126)	—	Brazil
de Jong *et al.* (2018)	—	1.6 (3/192)	2009–2012	Europe
Gindonis *et al.* (2013)	—	1.5 (2/135)	2005–2006	Finland
Haenni *et al.* (2011)	—	0.7 (1/139)	2007–2008	France
Huber *et al.* (2010)	—	1.4 (2/142)	2009	Switzerland
Jamali *et al.* (2014)	207	11.6 (5/43)	2008–2010	Iran
Jamali *et al.* (2015)	1035	13 (21/162)	2006–2013	Iran
Kamal *et al.* (2013)	35	9.1 (3/33)	2011–2012	Egypt
Kumar *et al.* (2010)	185	7.8 (10/128)	2007–2008	India
Kwon *et al.* (2005)	9055	6.0 (15/248)	1999, 2000, 2003	Korea
Lee (2003)	894	1.3 (12/265)	2001–2003	Korea
Li *et al.* (2015)	214	0.8 (1/121)	—	China
Luini *et al.* (2015)	—	9.2 (15/163)	2006–2013	Italy
Mekonnen *et al.* (2018)	—	0 (0/79)	2014–2016	Ethiopia
Moon *et al.* (2007)	3047	1.6 (13/835)	1997–2004	Korea
Oliveira *et al.* (2016)	552	32.3 (21/65)	—	Brazil
Pu *et al.* (2014)	450	49.6 (49/103)	2008	China
Qu *et al.* (2018)	—	4 (15/96)	2014–2017	China
Riva *et al.* (2015)	383	20.0 (7/35)	2012	Italy
Rola *et al.* (2015)	115	0 (0/71)	2009–2013	Poland
Ronco *et al.* (2018)	—	1.6 (1/63)	2016	Denmark
Ruegg *et al.* (2015)	—	0 (0/35)	2010	United States
Saini *et al.* (2012b)	—	0.1 (1/1810)	—	Canada
Shrivastava *et al.* (2018)	400	23.0 (57/248)	—	India
Song *et al.* (2016)	649	13.9 (23/165)	2011–2012	Korea
Turkyilmaz *et al.* (2010)	—	17.2 (16/93)	2002–2006	Turkey
Unnerstad *et al.* (2013)	8757	0.8 (4/534)	2010–2011	Sweden
Vanderhaeghen *et al.* (2010)	—	9.3 (11/118)	2006–2007	Belgium
Vyletělova *et al.* (2011)	724	1.7 (3/180)	—	Czech Republic, Slovakia

MRSA was defined as *mecA/mecC*-positive *S. aureus* strains.
MRSA, methicillin-resistant *S. aureus.*

TABLE 3. METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* PREVALENCE IN SINGLE DAIRY HERDS

References	*% MRSA prevalence (n = MRSA/n = number of cows)*	*Year(s) of collection*	*Country*
Hata (2016)	39.7 (31/78)	2005	Japan
Locatelli *et al.* (2017)	Farm A 4.8 (3/63), Farm B 60.0 (33/55)	2010	Italy
Magro *et al.* (2018)	12.5 (3/24)	—	Italy
Matyi *et al.* (2013)	5.3 (7/133)	—	United States
Falk (2018)	13.2 (139/1050)	2018	Israel
Schlotter *et al.* (2014)	28.6 (16/56)	2013	Germany
Silva *et al.* (2014)	11.0 (4/36)	—	Brazil
Spohr *et al.* (2011)	Farm A 7.5 (12/160), Farm B 16.7 (7/42), Farm C 5.1 (4/78)	2008	Germany
Unnerstad *et al.* (2018)	44 (11/25)	2012	Sweden

MRSA was defined as *mecA/mecC*-positive *S. aureus* strains.
MRSA, methicillin-resistant *S. aureus.*

2015; Cortimiglia *et al.*, 2016; Parisi *et al.*, 2016; Locatelli *et al.*, 2017; Ronco *et al.*, 2018). Studies from Brazil, China, and Israel also found LA-MRSA CC398 in mastitis milk samples (Silva *et al.*, 2014; Falk, 2018; Yi *et al.*, 2018). Furthermore, LA-MRSA CC398 was found in nasal swabs and in udder cleft swabs from dairy cows (Antoci *et al.*, 2013; Nemeghaire *et al.*, 2014; van Duijkeren *et al.*, 2014). The predominant LA-MRSA in Southeast Asia is multilocus sequence type 9 (ST9). It was also detected in milk samples (Wang *et al.*, 2012; Tenhagen *et al.*, 2018). In most studies, predominant MRSA strains were found within herds, suggesting a contagious transmission from cow to cow (Moon *et al.*, 2007; Holmes and Zadoks, 2011; Schlotter *et al.*, 2014; Luini *et al.*, 2015; Song *et al.*, 2016). However, a study from Italy reported a high heterogeneity of MRSA CC, *spa*-types, and genotypes within two dairy herds (Locatelli *et al.*, 2017). The authors concluded that the environment could act as a reservoir of these MRSA strains.

In 2011, a new *mecA* homolog ($mecA_{LGA251}$) was identified in isolates from milk samples that were phenotypically resistant to methicillin but tested negative for the *mecA* gene (Garcia-Alvarez *et al.*, 2011). This new *mecA* homolog is also known as *mecC* and is often carried by strains belonging to clonal complex 130 (CC130). Zoonotic transmission has been reported for *mecC*-CC130 MRSA (Harrison *et al.*, 2013). As of this writing, *mecC*-positive milk samples have been reported from Finland, the United Kingdom, Germany, and Sweden (Garcia-Alvarez *et al.*, 2011; Gindonis *et al.*, 2013; Unnerstad *et al.*, 2013; Paterson *et al.*, 2014; Schlotter *et al.*, 2014). In a review about MRSA in human and bovine mastitis, the authors additionally reported *mecC*-positive bovine *S. aureus* isolates from Portugal, Denmark, and France (Holmes and Zadoks, 2011). However, according to the authors, these findings had not been published and were based on personal communications.

Risk Factors for the Occurrence of MRSA in Dairy Herds

Improper milking hygiene

Proper milking hygiene and especially the use of post-milking teat disinfectants are important control strategies for *S. aureus* mastitis (Barkema *et al.*, 2006). In the past several decades, progressive use of milking hygiene procedures and other recommendations from the National Mastitis Council 5- and 10-point plan have led to a reduction in the prevalence of contagious mastitis pathogens in many countries (Makovec and Ruegg, 2003b; Barkema *et al.*, 2006; Ruegg, 2018).

A recent case study from Brazil reported a high MRSA prevalence (12.2%) in mastitis milk samples from one herd (Guimaraes *et al.*, 2017). The authors observed a lack of pre- and postdipping procedures, udder towels were used on more than one cow, and the use of gloves was inappropriate. On the farm with the highest overall MRSA prevalence (60%) in Italy, milkers were not using gloves (Locatelli *et al.*, 2017). In a study from Sicily, the milking hygiene score was negatively correlated with MRSA prevalence (Antoci *et al.*, 2013). The authors concluded that improper milking hygiene procedures may be a risk factor for MRSA transmission within dairy herds.

Contact with pigs

The most frequently detected bovine MRSA strain in Europe (CC398) was initially associated with pigs (Armand-Lefevre *et al.*, 2005; Voss *et al.*, 2005; Huijsdens *et al.*, 2006). In all studies on LA-MRSA CC398 in farm animals, pigs were most frequently affected and prevalence rates were up to 89% (Porrero *et al.*, 2012; Abreu *et al.*, 2019). Thus, it was assumed that pigs may transfer MRSA to bovines. A recent study on 844 dairy herds from Italy has not found any association between the MRSA status and the presence of any other animal species on the same farm (Cortimiglia *et al.*, 2016). In contrast, two studies from the Netherlands have found that 64% (9/14) and 47% (28/60) of MRSA-positive farms harbored cows and pigs (Olde Riekerink *et al.*, 2009; Tavakol *et al.*, 2012). Another Italian study has reported that both the number of pigs and the number of pig herds close to the dairy farms were associated with the MRSA status (Locatelli *et al.*, 2016). The authors have not only reported CC398 but also CC97 MRSA strains. An Italian study which analyzed CC97 MRSA isolates from pigs and cattle reported that all strains were very similar and that the detected clone spreads among pig and dairy cattle holdings in Italy (Feltrin *et al.*, 2016). One MRSA-affected dairy farm from Germany also housed dairy cows and pigs. The same *spa*-type (t011) was found in the dairy cows and in the pig stall environment. The authors concluded that transmission might occur between the two livestock holdings (Spohr *et al.*, 2011). Therefore, certain MRSA strains, especially those of CC398, can probably spread between pigs and cows. Possible routes of transmission between the stables are dust (wind), rodents, people working with both species, and equipment used in both parts of the farm (van de Giessen *et al.*, 2009; Graveland *et al.*, 2010; Visciano *et al.*, 2014).

Humans carrying MRSA

Epidemiological investigations have suggested that sequence types of bovine and human *S. aureus* strains are usually different, and the risk of zoonotic and reverse zoonotic transmission is low (Holmes and Zadoks, 2011; Fitzgerald, 2012; Fluit, 2012). This seems to be different for MRSA, where the majority of isolates are considered LA-MRSA strains that infect or colonize both, humans and cattle.

Additionally, several reports of community and health care-associated MRSA (CA-/HA-MRSA) strains in dairy cows were published (Table 4). A case report from Australia has found a CA-MRSA strain (ST1, t127-IV), also known as WA-MRSA-1, in a milk sample of a subclinical mastitis case (Abraham *et al.*, 2017). According to the authors, WA-MRSA-1 is one of the most prevalent CA-MRSA strains circulating in Australia. Whole-genome sequencing has proved that both MRSA strains carried similar resistance and virulence genes. The authors concluded that transmission might have occurred from humans to the dairy cow. Unfortunately, the authors could not obtain samples from the farm personnel to confirm this hypothesis. Molecular analysis of human and bovine ST1-MRSA stains in Italy showed several human-associated genetic features in bovine isolates (Alba *et al.*, 2015). Other cases of CA-MRSA ST1, t127 in cattle, were reported from Germany, Italy, Switzerland, and Hungary (Juhasz-Kaszanyitzky *et al.*, 2007; Huber *et al.*, 2010; Pilla *et al.*, 2012; Tenhagen *et al.*, 2018). The authors from Italy assumed that humans were probably the source of infection, since the infected cow was kept on a closed farm (Pilla *et al.*, 2012). HA-MRSA was found in dairy cows in

Table 4. Reports of Community and Health Care-Associated Methicillin-Resistant *Staphylococcus aureus* Isolates in Samples from Dairy Cows

References	*MLST/spa-type/SCC*mec *type of MRSA*	*Year(s) of collection*	*Country*
Abraham *et al.* (2017)	ST1/t127/IV	2015	Australia
Bardiau *et al.* (2013)	ST8/t008/IV	2005–2008	Belgium
Haenni *et al.* (2011)	ST5/t002/I	2007–2008	France
Haran *et al.* (2012)	ST8/t121/IVa, ST5/-/II	2009	United States
Hata *et al.* (2010)	ST5/t002/II, ST89/t5266/IIIa	1998–2005	Japan
Huber *et al.* (2010)	ST1/t127/IV	2009	Switzerland
Juhasz-Kaszanyitzky *et al.* (2007)	ST1/t127/IV	2002–2004	Hungary
Luini *et al.* (2015)	ST1/t127/IV, ST8/t3092/V	2006–2013	Italy
Magro *et al.* (2018)	ST22/-/-	—	Italy
Monecke *et al.* (2007)	ST8/t068/-	—	Switzerland, Germany
Nam *et al.* (2011)	ST72/t324/IVa	2003–2009	Korea
Parisi *et al.* (2016)	ST1/t127/IVa, ST5/t688/V, ST8/-/IVa, V	2012–2013	Italy
Pilla *et al.* (2012)	ST1/t127/IV	—	Italy
Song *et al.* (2016)	-/t148/IVa	2011–2012	Korea
Tenhagen *et al.* (2018)	ST1/t127/-, ST22/t790/-	2014	Germany
Turkyilmaz *et al.* (2010)	ST239/t030/III, ST8/t190/IV	2002–2006	Turkey

MRSA was defined as *mecA/mecC*-positive *S. aureus* strains.
MLST, multilocus sequence typing; MRSA, methicillin-resistant *S. aureus*; SCC*mec*, staphylococcal cassette chromosome *mec*.

Germany (ST22), Japan (ST5), the United States (ST5), and Turkey (ST239) (Hata *et al.*, 2010; Turkyilmaz *et al.*, 2010; Haran *et al.*, 2012; Tenhagen *et al.*, 2018). In Korea CA-MRSA (ST72, t324-IVa) and HA-MRSA (t148-IVa) were detected in milk samples (Nam *et al.*, 2011; Song *et al.*, 2016). A study from France reported the human-associated epidemic Geraldine-MRSA clone (ST5, t002-I) in a bovine milk sample (Haenni *et al.*, 2011). In conclusion, CA- and HA-MRSA may be transferred to dairy cows. In light of the increasing numbers of CA- and HA-MRSA isolates in samples from cattle, the relevance of reverse zoonotic MRSA transmission might be underestimated.

Production system

A study from the United States has reported that *S. aureus* isolates from organic farms were phenotypically more susceptible to antimicrobials than isolates from conventional farms (Tikofsky *et al.*, 2003). In contrast, a study from Denmark has not found a significant difference in susceptibility to penicillin between *S. aureus* isolates from organic and conventional farms (Bennedsgaard *et al.*, 2006).

Currently, only two MRSA (*mecA/mecC*) prevalence studies have differentiated between organic and conventional production systems. One study from the United States tested BTM from 192 organic and 100 conventional farms for the *mecA/meC* gene (Cicconi-Hogan *et al.*, 2014). The authors only found one MRSA isolate in all farms and concluded that MRSA prevalence is low independent of the production system. The other study included 372 conventional and 303 organic BTM samples from Germany (Tenhagen *et al.*, 2018). The MRSA prevalence was lower in organic herds (1.7%) than in conventional herds (9.7%). Consequently, there is some evidence that cows from conventional farms are more likely to carry MRSA than cows from organic farms.

Herd size

Two studies reported positive correlations between herd size and MRSA prevalence. In Germany, the prevalence of MRSA in BTM was higher on conventional farms with a larger herd size than on small farms (Tenhagen *et al.*, 2018). An Italian study found the highest *S. aureus* prevalence (68.5%) in BTM samples from Sondrio province, where farms are small (median value 20 animals) (Cortimiglia *et al.*, 2016). In contrast, the highest MRSA prevalences of 10.8% and 6.4% were reported from the provinces of Cremona and Lodi, where the median herd size was the highest in this study (325 and 278 cows/herd, respectively). In another Italian study, the average size of dairy herds tended to be positively correlated with MRSA status ($p = 0.08$) (Locatelli *et al.*, 2016). On larger farms, more cows contribute to the BTM, increasing the likelihood of a positive BTM with a given cow level prevalence. Higher numbers of trading contacts and a higher use of third-generation cephalosporins may also contribute to a higher MRSA prevalence in BTM from large dairy herds (Saini *et al.*, 2012a). However, smaller farms are probably more likely to keep multiple animal species, including pigs. This is also considered a risk factor for the presence of MRSA in a dairy herd.

Methicillin-Resistant Coagulase-Negative-Staphylococci

Coagulase-negative staphylococci (CoNS) are a diverse group of predominantly opportunistic pathogens. In several studies, CoNS were the most frequently detected organisms from milk samples (Pitkälä *et al.*, 2004; Sampimon *et al.*, 2009; Tenhagen *et al.*, 2009; Oliveira *et al.*, 2016). Molecular studies suggest that CoNS carry fewer virulence genes than *S. aureus* and are therefore considered less pathogenic (Åvall-Jääskeläinen *et al.*, 2018). In China, 73% (82/112) of non-aureus staphylococci carried the *mecA* gene and MRSA prevalence was 4% (15/96) (Qu *et al.*, 2018). A study from the United States has reported 11 methicillin-resistant coagulase-negative staphylococci (MR-CoNS) in BTM from 288 farms and just 1 single MRSA isolate (Cicconi-Hogan *et al.*, 2014). In contrast, in 3047 mastitis milk samples from Korea, the authors reported 12 MR-CoNS and 13 MRSA

isolates (Moon *et al.*, 2007). This would be in line with the lower virulence of the MR-CoNS. The last VetPath study from Europe reported that 7 of 165 CoNS isolates from mastitis milk samples carried the *mecA* gene (4.2%) and 1.6% (3/192) of *S. aureus* isolates were classified as MRSA (de Jong *et al.*, 2018). In Finland, two studies reported that 5.2% (17/324) and 1.8% (2/110) of the CoNS isolates were *mecA* positive and MRSA prevalence among *S. aureus* isolates was 1.5% (2/135) (Gindonis *et al.*, 2013). A study from Portugal did not find MRSA but 9.3% (19/204) of mastitis milk samples were positive for MR-CoNS (Seixas *et al.*, 2014). In conclusion, MR-CoNS have been detected in MRSA affected dairy herds and the prevalence of methicillin resistance was generally higher than in *S. aureus*.

A study from Belgium reported that SCC*mec* types in bovine MR-CoNS ($n = 101$) differed from those mostly detected in LA-MRSA CC398 (Vanderhaeghen *et al.*, 2013). The authors assumed that the SCC*mec* of MR-CoNS is probably not a reservoir of resistance determinants for LA-MRSA CC398. However, it is well known that resistance genes can be transferred between staphylococcal species (Morikawa *et al.*, 2012; Chlebowicz *et al.*, 2014; Ray *et al.*, 2016). The *in vivo* transfer of SCC*mec* was the most probable explanation for identical SCC*mec* in *S. aureus* and *Staphylocccus epidermidis* in an infected patient, although transmission could not be reproduced *in vitro* (Bloemendaal *et al.*, 2010). *In vitro*, the transfer of SCC*mec* was achieved through transformation (incorporation of DNA from the environment) (Morikawa *et al.*, 2012), through plasmids (Ray *et al.*, 2016), conjugation (sexual transfer) (Tsubakishita *et al.*, 2010), and transduction (bacteriophage transfer) (Chlebowicz *et al.*, 2014). All these studies were performed under laboratory conditions. To the best of our knowledge, it remains unclear which mechanism(s) of SCC*mec* transfer occur *in vivo*. In conclusion, MR-CoNS could act as a reservoir of resistance genes that may be transferred to MSSA in dairy cows. The role of SCC*mec* transfer for the development of new MRSA strains needs to be further investigated.

The Amount of Antibiotics Used on Dairy Farms

The use of antibiotics is associated with the development of antibiotic resistance (Chantziaras *et al.*, 2014). Every time bacteria are exposed to antimicrobial agents, selection pressure will cause antibiotic resistance to increase (Lam *et al.*, 2014). A meta-analysis reported a significant association between antimicrobial exposure and the number of MRSA isolates in humans (Tacconelli *et al.*, 2008).

For dairy cows, mastitis is the leading cause of antibiotic treatment. Blanket DCT with long-acting β-lactam antimicrobials, especially cloxacillin, is still commonly applied to prevent and cure intramammary *S. aureus* infections (Oliver *et al.*, 2011; Saini *et al.*, 2012a; Oliveira *et al.*, 2016). Therefore, it is hypothesized that the large-scale use of β-lactams in dairy cows is a possible risk factor for the selection of new MRSA strains (Saini *et al.*, 2012c).

A study from Germany found a lower MRSA prevalence in organic herds (1.7%) than in conventional herds (9.7%) (Tenhagen *et al.*, 2018). Organic farmers are considered to use fewer antibiotics. In a study from the Netherlands, veal calves were more often MRSA carriers when treated with antibiotics (Graveland *et al.*, 2010).

Unfortunately, most studies that included the amount of antibiotics used on dairy farms only performed phenotypic resistance testing and did not detect the *mecA*/*mecC* gene. This matters, since phenotypic testing was shown to lead to false-negative (Pu *et al.*, 2014; Guimaraes *et al.*, 2017) and false-positive results in previous studies (Cicconi-Hogan *et al.*, 2014; da Costa Krewer *et al.*, 2015; Li *et al.*, 2015; de Jong *et al.*, 2018; Wang *et al.*, 2018). In Thailand, milk samples from 78 cows on 18 farms were tested for phenotypic oxacillin resistance (Suriyasathaporn *et al.*, 2012). The authors reported higher numbers of methicillin resistant staphylococci on farms with high antibiotic use (21%) than on farms with normal use of antibiotics (5.9%). High antibiotic use was defined as more than two treatment periods per cow per year and normal use as no more than two treatment periods per cow per year. One study from Canada has found a positive correlation between intramammary and systematically administered penicillin treatments and phenotypic penicillin resistance in 89 dairy herds (Saini *et al.*, 2012c).

A study from the United States included 2778 mastitis isolates for phenotypic antibiotic susceptibility testing over a 6-year period, from 1994 to 2000. The proportion of isolates, which were phenotypically susceptible to β-lactam antimicrobials, did not change during the period (Erskine *et al.*, 2002). Another study from the United States has not reported a higher proportion of *S. aureus* isolates that were phenotypically resistant to any antimicrobial drug (Makovec and Ruegg, 2003a). In a literature review about the impact of antibiotic use in dairy cows on antimicrobial resistance, the authors concluded that there is no evidence for increasing resistance rates due to antibiotic treatment (Oliver *et al.*, 2011).

In conclusion, there is an ongoing debate about the role of antimicrobial exposure as a risk factor for the occurrence of MRSA in dairy cows. It was suggested that antimicrobial resistance is low in milk because the total number of bacteria in the udder is low in comparison to the intestinal tract, skin, or mucous membranes. For this reason, resistance levels through intramammary treatment might be lower than in other parts of the body after oral or parenteral application of antibiotics (Lam *et al.*, 2014).

Association of MRSA with a High Somatic Cell Count in Milk

The somatic cell count is the number of cells present in milk (cells/mL). Beside some epithelial cells, the majority of somatic cells are cells from the immune system (Harmon, 1994). Therefore, a higher somatic cell count is considered a reflection of an inflammatory response in the mammary gland. The most reliable somatic cell count cutoff value for mastitis detection is between 200,000 and 250,000 cells/mL (Laevens *et al.*, 1997; Schepers *et al.*, 1997; Schukken, 2007).

A German study has reported that quarters harboring MRSA had a higher somatic cell count than other quarters (Spohr *et al.*, 2011). In a case report about MRSA in a Brazilian dairy herd, the bulk milk somatic cell count was 628,000 cells/mL (Guimaraes *et al.*, 2017). In Sicily, a negative correlation between somatic cell count and MRSA status in BTM from 45 dairy farms was reported (Antoci *et al.*, 2013). A study from Italy detected higher somatic cell counts (286,000 ± 212,000 cells/mL) in BTM from MRSA-affected farms in comparison to farms with negative test

results (236,000 ± 231,000 cells/mL) (Locatelli *et al.*, 2016). However, this difference was not significant ($p = 0.38$). Two Italian studies sampled milk from MRSA-infected cow(s) continuously over the entire lactation. The somatic cell count in MRSA-infected quarters fluctuated between 300,000 and 6,000,000 cells/mL in one study and between 1000 and 1,800,000 cells/mL in the other study (Pilla *et al.*, 2012; Magro *et al.*, 2018). In one study, the authors reported that fluctuation was not related to the shedding of MRSA (Pilla *et al.*, 2012). A Swedish case study reported somatic cell counts between 12,000 and 2,885,000 cells/mL in MRSA-positive milk samples (Unnerstad *et al.*, 2018). In China, 5 MRSA isolates have been reported among 121 quarter milk samples. All 5 MRSA were isolated from clinically healthy cows with a somatic cell count <300,000 cells/mL (Bao *et al.*, 2016). In a case report from Japan, the authors have reported a low bulk tank somatic cell count of 114,000 cells/mL in a MRSA-affected herd (Hata, 2016). The somatic cell count in a German dairy herd with high MRSA prevalence was even lower with 51,600 cells/mL (Schlotter *et al.*, 2014). Thus, a higher somatic cell count in milk is probably not a reliable indicator for the occurrence of MRSA in dairy herds.

Additional Risk Factors for Udder Infections Caused by *S. aureus* in Dairy Cows That Have Not Been Addressed in Studies on MRSA

Some studies have suggested that older cows are more likely to be *S. aureus* infected (Pyörälä and Pyörälä, 1998; Barkema *et al.*, 2006). Moreover, a study found higher rates of phenotypic penicillin resistance in animals from the third and following lactations, than in animals from the first and second lactation (Sol *et al.*, 2000). In addition, a larger mammary gland size was shown to be predisposing for *S. aureus* infections and hind quarters were more frequently affected (Deluyker *et al.*, 2005). Furthermore, it has been known that purchasing infected replacement heifers and people that have visited many farms per day (e.g., veterinarians, artificial insemination technicians, and cattle traders) might introduce new *S. aureus* strains into dairy herds (Middleton *et al.*, 2002). Moreover, some studies have found multiple different *S. aureus* strains within dairy herds, suggesting that in some cases *S. aureus* might be regarded as a sporadic environmental pathogen (Sommerhäuser *et al.*, 2003; Zadoks *et al.*, 2011). *S. aureus* has been detected in environmental samples, such as, flies, bedding materials, and feedstuff (Roberson *et al.*, 1998; Capurro *et al.*, 2010; Zadoks *et al.*, 2011). Further studies are needed to confirm these findings for MRSA in dairy farms.

Conclusion

The risk factors for the transmission of MRSA into dairy herds are direct or indirect contact with pigs and humans carrying MRSA. Within dairy herds, MR-CoNS may transfer resistance genes to MSSA. Moreover, improper milking hygiene procedures enhance the spread of MRSA within herds as is well known for MSSA. There is some evidence that conventional dairy farms and farms with a larger herd size are more often affected by MRSA. The association of antimicrobial exposure and MRSA prevalence in dairy herds needs to be further investigated. High amounts of β-lactam antibiotics have been used for dry cow treatment and mastitis therapy on dairy farms. Nevertheless, MRSA prevalence is low in dairy cows. Furthermore, it is not known whether additional risk factors for *S. aureus* transmission in dairy herds differ from those of MRSA. According to our findings, a higher somatic cell count in milk is probably not a reliable indicator for the occurrence of MRSA in dairy herds.

The risk of foodborne zoonotic MRSA infections through consumption of milk seems to be low. Milk is usually heat treated before marketing and consumption and MRSA prevalence is low in milk from dairy cows. However, MRSA prevalence should be carefully monitored, since some studies suggest increasing levels of resistance.

In veterinary medicine, MRSA emerge as mastitis pathogens in dairy cows and spread within herds. Dry cow treatment with β-lactam antibiotics, as an important part of *S. aureus* control programs, is probably ineffective in curing MRSA infections. Therefore, segregation and culling of infected cows often remains the only option for removing MRSA from dairy herds. In conclusion, we stress the need for a continuous MRSA monitoring in dairy herds and the development of MRSA prevention strategies.

Disclosure Statement

No competing financial interests exist.

Funding Information

This study was carried out in the framework of the project #1HealthPREVENT, funded by the German Federal Ministry of Education and Science, Grant No. 01KI1727C.

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3.2 Publication 2

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The occurrence and distribution of livestock-associated methicillin-resistant *Staphylococcus aureus* ST398 on German dairy farms

https://doi.org/10.3168/jds.2020-18958

Journal of Dairy Science

December 2020; 103(12);11806-11819.

J. Dairy Sci. 103 1806–11819
https://doi.org/10.3168/jds.2020-18958

The occurrence and distribution of livestock-associated methicillin-resistant *Staphylococcus aureus* ST398 on German dairy farms

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ABSTRACT

The objective of this study was to investigate the occurrence and distribution of methicillin-resistant *Staphylococcus aureus* (MRSA) on 20 German dairy farms. Farms were selected based on previous MRSA reports from phenotypic susceptibility testing of mastitis pathogens. Samples were collected from predefined groups of cows, young stock, farm personnel, and the environment. A high MRSA-positive test rate was detected in swab samples from milk-fed calves (22.7%; 46/203). In postweaning calves, the MRSA-positive test rate was 9.1% (17/187). From prefresh heifers, both nasal swabs and udder cleft swabs were collected if possible. Including both sample types, the MRSA-positive test rate in prefresh heifers was 13.0% (26/200). The positive test rate was 8.9% (17/191) in nasal swabs and 6.5% (11/170) in udder cleft swabs. In quarter milk samples (QMS), the MRSA-positive test rate was 2.9% (67/2347), and on cow level, 7.9% (47/597) of the dairy cows were affected. Among all cows included in this study, the geometric mean of somatic cell counts was higher in QMS that carried MRSA (345,000 cells/mL) in comparison to all QMS (114,000 cells/mL). No differences in parity or the affected mammary quarter position on the udder were observed among the 47 infected cows. Methicillin-resistant *S. aureus* was also detected in boot swab samples (dust), teat liners, and in suckers from automatic calf feeders. All isolates belonged to livestock-associated sequence type 398 and most common staphylococcal protein A (*spa*)-types were t011 and t034. Most isolates harbored the staphylococcal cassette chromosome mec (SCC*mec*)-type V, with the exception of some isolates with SCC*mec*-type IVa on 1 farm. Similar MRSA genotypes in samples from humans and dairy cows underline the possible zoonotic and reverse-zoonotic transmission of livestock-associated MRSA strains from dairy farms. Similar MRSA genotypes in pig and cattle barns were detected on only 1 of 5 farms that kept both cattle and pigs. Similar MRSA *spa*-types were detected in samples from different sources (dairy cows, young stock, environment, and humans), suggesting a possible contagious transmission on some of the farms. Sporadically, up to 3 different MRSA *spa*-types were detected in QMS from the respective farms. On MRSA-affected farms, improper milking hygiene procedures and elevated bulk-tank milk somatic cell counts (>250,000 cells/mL) were observed. The occurrence of livestock-associated MRSA ST398 in different samples from dairy farms, and especially in young calves, should be considered for future MRSA-monitoring programs and biosecurity guidelines.

Key words: livestock-associated methicillin-resistant *Staphylococcus aureus*, antimicrobial resistance, dairy cattle

INTRODUCTION

In dairy cattle herds, *Staphylococcus aureus* is the most important contagious mastitis-causing pathogen, and it negatively affects animal welfare, milk quality, and dairy-farm profit (Heikkilä et al., 2018; Ruegg, 2018). Methicillin-resistant *S. aureus* (**MRSA**) carries a *mecA* or *mecC* gene, which mediates resistance against β-lactam antibiotics. Since the 1960s, MRSA has been a major human health burden as a nosocomial pathogen (Köck et al., 2010). In 2005, a new group of MRSA that is associated with animals was detected, known as livestock-associated MRSA (**LA-MRSA**; Armand-Lefevre et al., 2005; Huijsdens et al., 2006). The predominant LA-MRSA strain worldwide is sequence type (**ST**) 398, except in Asia (where ST9 is more common; Aires-de-Sousa, 2017). In European pig holdings, LA-MRSA ST398 is widespread and occasionally causes infections in humans that range from mild skin infections to more serious invasive infections, and even death (Goerge et al., 2017; Abreu et al., 2019). Molecular analysis of many LA-MRSA ST398 strains showed low numbers of virulence factor–associated genes (Argudín et al., 2011; Hansen et al., 2019). In Germany, about 3.7% of all

Received May 26, 2020.
Accepted July 28, 2020.
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human clinical MRSA isolates, tested at the national reference center for staphylococci, were associated with LA-MRSA ST398 (Layer et al., 2019). However, in regions with high livestock density and in countries with low numbers of healthcare-associated MRSA infections, the proportion of LA-MRSA ST398 among isolates from humans was up to 35% (DANMAP, 2016; van Alen et al., 2017).

Although methicillin-susceptible *S. aureus* (**MSSA**) isolates from dairy cows are usually host specific, isolates from ST398 can spread between different species (Holmes and Zadoks, 2011; Leuenberger et al., 2019). The transmission of LA-MRSA between animals and humans has been reported in several studies, including studies from dairy farms (Cuny et al., 2015; Locatelli et al., 2017).

Worldwide, MRSA has been detected in samples from dairy cows (Schnitt and Tenhagen, 2019). The majority of LA-MRSA ST398 has been detected in milk samples from Europe, but also from Israel, China, and Brazil (Guimarães et al., 2017; Falk, 2018; Yi et al., 2018). In Europe, MRSA prevalence in bulk-tank milk (**BTM**) was 3 to 10%, with ST398 being the most common strain (Cortimiglia et al., 2016; Tenhagen et al., 2018; Hansen et al., 2019). Individual mastitis outbreaks in dairy herds caused by LA-MRSA ST398 have been reported, as well (Locatelli et al., 2017; Falk, 2018). Studies from South Korea and Europe have reported that MRSA prevalence in dairy herds might be increasing in some regions (Song et al., 2016; Tenhagen et al., 2018).

From an animal health perspective, *S. aureus* is a challenging mastitis-causing pathogen because cure rates are generally low (Ruegg, 2018). Broad spectrum β-lactam resistance in MRSA further minimizes treatment options, especially because dry cow therapy with cloxacillin, which is widely recommended for MSSA therapy, is probably ineffective (Makovec and Ruegg, 2003; Saini et al., 2012). Possible human health hazards, limited treatment options, and MRSA reports from around the world stress the need for research on MRSA in dairy herds. To date, few studies reported the occurrence of MRSA in different sample types from single dairy farms, indicating that MRSA detection in BTM may just be the tip of the iceberg. Therefore, we aimed to systematically analyze the occurrence and spread of MRSA in preselected German dairy farms.

MATERIALS AND METHODS

Recruitment and Herd Selection

Between November 2018 and December 2019, 20 dairy herds from different regions in Germany were visited. All herds had tested positive for MRSA in previous years, mostly during routine testing of bacteria-causing mastitis. Suspected farms were identified by udder health laboratories and veterinary practitioners that performed phenotypic susceptibility testing and detected oxacillin-resistant *S. aureus* isolates.

Sampling

From 20 dairy farms, 3,782 samples were analyzed. In total, 2,347 quarter milk samples (**QMS**) from 597 cows and 19 BTM samples were collected for somatic cell count measurements and bacterial culture. Nasal swabs from 201 milk-fed calves, 187 postweaning calves, and 191 prefresh heifers were collected for bacterial culture. From the prefresh heifers, 170 udder cleft swabs were additionally collected. Human samples (n = 14) were obtained from 7 farms. From each dairy farm, 1 dust sample from the dairy barn and 1 swab sample from teat liners were analyzed. Teat liners were sampled after cluster disinfection, if cluster disinfection was performed. On farms that additionally housed pigs, dust samples from the pig barns were collected as well. On farms that used automatic calf feeders, a swab sample from the sucker was collected. On each farm, approximately 10 primiparous, 10 multiparous, and 10 high-risk cows were selected for the collection of QMS. The 10 primiparous and 10 multiparous cows were randomly selected during the milking process in accordance with the farm personnel. The high-risk cows had current high somatic cell counts in QMS or previous MSSA or MRSA reports. Therefore, we expected that these cows have a higher probability to carry MRSA. The QMS were collected aseptically by a trained veterinarian according to the guidelines of the German Veterinary Medical Association (DVG, 2009). Nasal swab samples were collected from milk-fed calves (n = 10) and postweaning calves (n = 10). The nasal swab was inserted and rotated in both nasal vestibules. From prefresh heifers, nasal swabs (n = 10) and udder cleft swabs (n = 10) were collected if possible. Due to missing head locks in some heifer barns, collection of udder cleft swabs was not always possible. Sampling procedures were performed in accordance with the German legislation. For the collection of nasal swab samples, udder cleft swab samples, and QMS from cattle, no ethical approval was required according to the German Animal Welfare Act (TierSchG) because they were carried out as part of a diagnostic investigation in the suspect farms. All samples were transported to the laboratory in a mobile cooling box within 1 d.

Questionnaire and On-Farm Observations

A structured questionnaire was used to collect data on general farm management, MRSA history, other animal species present on the farm, biosecurity measures, and milking parlors or automatic milking systems (**AMS**). Furthermore, milking hygiene procedures were observed during the milking process and results were documented in the questionnaire. The application of the postdip and spray was evaluated by the paper towel test. A clean paper towel was placed on the teat ends, and wet spots indicated a coverage with dipping and spraying solution. Further data from monthly milk recordings were obtained from 17 farms, and the BTM somatic cell count history was analyzed using the herd management software HERDE (dsp-Agrosoft GmbH, 14669, Ketzin, Germany)

Somatic Cell Count Measurements

Somatic cell counts of all milk samples (QMS and BTM) were measured within 48 h after collection by DeLaval cell counter DCC according to the manufacturer's instructions (DeLaval International, SE-147 21 Tumba, Sweden). Cut-off values for high somatic cell counts were >150,000 cells/mL in primiparous cows and >250,000 cells/mL in multiparous cows.

Isolation of MRSA

Milk and swab samples were examined using a double selective-enrichment method. Each swab sample (COPAN Diagnostics Inc., Murrieta, CA) and 1 mL of each QMS was incubated in 10 mL (swab samples) or 9 mL of Mueller Hinton broth supplemented with 6.0% NaCl for 24 ± 2 h at 37°C. Of this pre-enrichment broth, 1 mL was transferred into 9 mL of tryptic soy broth supplemented with 3.5 mg/L of cefoxitin and 50 mg/L of aztreonam. After incubation for 24 ± 2 h at 37°C, 50 µL of the selective-enrichment broth was plated onto mannitol salt agar (**MSA**) containing 4 mg/L of cefoxitin and incubated for 24 ± 2 h at 37°C. From each BTM sample, 3 separate batches of 1 mL of BTM and 9 mL of Mueller Hinton broth with 6.0% NaCl were incubated for 48 ± 2 h and streaked on MSA-Cefoxitin agar. Colonies from MSA-Cefoxitin agar plates (BTM and QMS) were subcultured on sheep blood agar (Oxoid GmbH, 46483, Wesel, Germany) and further identified by a MALDI-TOF mass spectrometer according to the manufacturer's instructions (Bruker Scientific LLC, Billerica, MA). Colonies were spotted on the MALDI-TOF target via direct transfer method (Cameron et al., 2017) and covered with 1.0 µl of α-Cyano-4-hydroxycinnamic acid (Bruker Scientific LLC). According to the manufacturer's recommendations, the threshold score for acceptable *S. aureus* species identification was 2.000. The reference database was provided by Bruker (MBT-BDAL-8468).

Molecular Typing

The DNA extraction of presumptive MRSA isolates was done by thermal lysis as previously described (Schouls et al., 2009). Extracted DNA was stored at −20°C until further processing. All presumptive MRSA isolates were confirmed by a real time multiplex PCR targeting the *tuf* gene (specific for staphylococci), the *nuc*-gene (specific for *S. aureus)*, the resistance gene *mecA*, and the *pvl* gene. The *pvl* gene encodes for the pathogenicity factor Panton-Valentine leucocidin, which is associated with community-acquired infections in humans (Kilic et al., 2010; Fosheim et al., 2011). If MRSA isolates were phenotypically resistant to cefoxitin but did not carry the *mecA* gene, they were further tested for the *mecC* gene (García-Álvarez et al., 2011). For *mecA*-carrying isolates, staphylococcal cassette chromosome mec (**SCC*mec***) types were determined using a multiplex PCR (Zhang et al., 2005). All MRSA isolates were further typed according to their polymorphic 24-base pair variable-number tandem repeat within the 3′ coding region of the staphylococcal protein A (***spa***) gene (Shopsin et al., 1999). Sequencing of purified *spa*-gene products was performed by Eurofins laboratories (Eurofins Genomics, 85560, Ebersberg, Germany). *Spa*-types (**t**) and associated ST were assigned using Ridom Spa Server (https://spaserver.ridom.de/) and Fortinbras *spa*Typer (http://spatyper.fortinbras.us/). All MRSA isolates were prepared as glycerol stocks and stored at −80°C.

Recruitment and Molecular Analysis of Human Samples

Recruitment of farm personnel was based on direct contact during the farm visits. All humans voluntarily agreed to participate by signing a declaration of consent. Sampling was performed by self-collection of nasal swabs. The eSwab system from MAST Diagnostica (Mast Diagnostica GmbH, 23858, Reinfeld, Germany) was used for taking swab samples from both nostrils by 1 and the same swab. Feasibility of self-collection was reported previously (Akmatov et al., 2014). All human swab samples (n = 14) were sent to the Robert Koch Institute (RKI, 38855, Wernigerode, Germany) for further analysis. After nonselective-enrichment of the swabs in cation-adjusted Mueller Hinton broth, aliquots were streaked on CHROMagar MRSA from Becton Dickinson (Becton Dickinson GmbH, 69126 Heidelberg,

Germany) and in parallel on Mueller Hinton blood agar plates from Oxoid. After incubation at 37°C for 24 h, 1 suspicious colony was subcultured on sheep blood agar. Confirmation of *S. aureus* was performed by demonstration of the clumping factor and additionally by the tube coagulase test. In the case of negative results, we performed PCR for the *S. aureus* specific region of *tuf* gene by use of primers and PCR conditions according to reference (Martineau et al., 2001). Studying nasal MRSA colonization of humans occupationally exposed to livestock was approved by the ethical committee of the medical faculty of Magdeburg University (#33/14).

Statistical Analysis

Data were expressed as frequencies and percentages. The positive test rate and 95% confidence interval (**CI**) of MRSA-affected samples from the specific populations were calculated and expressed as percentage (positive test rate = number of MRSA-positive samples/number of all samples from the specific population). The MRSA-positive test rate in primiparous and multiparous cows was compared using a Pearson χ^2 test. A hierarchical generalized linear mixed model was used to determine the effect of quarter position (left front, right front, left hind, and right hind), somatic cell count, and cow group (primiparous, multiparous, and high-risk group) on the positive test rate of MRSA in QMS. Farm number (no.) and cow number nested in farm was included as a hierarchical random variable. Odds ratios (**OR**) with 95% CI were calculated. The OR were considered significant if the underlying P-value was smaller than 0.05 ($P < 0.05$). The OR were interpreted as the effect of quarter position, somatic cell count, and cow group on the occurrence of MRSA in QMS. Statistical analysis was carried out in SPSS version 26.0 (IBM Corp., Armonk, NY).

RESULTS

MRSA in Dairy Cows

Herd size ranged from 26 to 970 cows per farm. The majority of farms kept Holstein Friesian cattle (n = 15), 4 farms had Simmental cattle, and 1 farm kept Angler cattle. Eight farms used AMS, 11 farms used milking parlors, and 1 farm used both AMS and a milking parlor. The detection of MRSA in different samples from the 20 preselected German dairy farms is presented in Table 1. On quarter level, 2.9% (67/2347; 95% CI: 2.2–3.6%) of all QMS tested positive for MRSA. In 13 cows, more than one-quarter tested positive for MRSA. The occurrence of MRSA in QMS from the 3 preselected groups of cows is presented in Table 2. No difference was observed in the MRSA-positive test rate from QMS of randomly selected first-lactation cows (1.7%, 11/655; 95% CI: 0.8–3.0%) and multiparous cows (2.1%, 23/1083; 95% CI: 1.4–3.2%; $P = 0.511$). In total, 5.5% (33/603; 95% CI: 3.8–7.6%) of QMS in the high-risk group carried MRSA. Within the high-risk group, 137 of 603 QMS were obtained from primiparous cows, and 466 of 603 QMS were from multiparous cows.

Positive QMS were obtained from 47 of 597 cows (7.9%; 95% CI: 5.5–10.3%). The highest proportion of MRSA-carrying preselected cows within herds was 43% (13/30). On cow level, 13.5% (21/156; 95% CI: 8.5–19.8%) of high-risk cows, 6.1% (17/277; 95% CI: 3.6–9.6%) of randomly selected multiparous cows, and 5.5% (20/152; 95% CI: 2.5–10.1%) of primiparous cows tested positive for MRSA. Regarding the cattle breeds, 43 Holstein Friesian cows from 11 farms and 4 Simmental cows from 1 farm carried MRSA. In the Angler cattle herd, MRSA was detected in the BTM but not in QMS from preselected cows.

The average somatic cell count (geometric mean) was 345,000 cells/mL in QMS that carried MRSA, and 114,000 cells/mL in all QMS. In MRSA-positive QMS from primiparous cows, 47% (9/19) showed high somatic cell counts (>150,000 cells/mL). In multiparous cows, somatic cell counts were high (>250,000 cells/mL) in 74% (35/47) of MRSA-carrying QMS.

Results from the generalized linear mixed model analysis are presented in Table 2. The QMS from high-risk cows were 3 times more likely to carry MRSA (OR = 2.9; 95% CI: 1.4–6.3; $P = 0.006$) than randomly selected primiparous or multiparous cows. The QMS with high somatic cell counts (>150,000 cells/mL in primiparous cows, and >250,000 cells/mL in multiparous cows) were 6 times more likely to carry MRSA (OR = 6.2; 95% CI: 3.5–10.9; $P = 0.000$) compared with QMS with low somatic cell counts (<150,000 or <250,000 cells/mL in primiparous and multiparous cows, respectively). The quarter position (left front, right front, left hind, and right hind) was not associated with MRSA-positive test rate ($P > 0.05$). The variance of the random variable showed evidence for significant variation between farms in terms of MRSA-positive QMS ($P = 0.025$).

On 10 of 12 farms with MRSA detection in QMS, MRSA was detected in BTM. On 2 farms, MRSA was detected in BTM but not in QMS from selected cows. The somatic cell counts in BTM from previous milk test recordings (3 mo) are presented in Table 3. On 9 of 14 farms with MRSA detection in milk, the average BTM somatic cell count (geometric mean) from the last 3 mo before our visit was >250,000 cells/mL, and 2 farms did not report milk test recordings. The highest BTM somatic cell count (geometric mean) from the last

Table 1. Numbers of methicillin-resistant *Staphylococcus aureus* (MRSA) in different samples from 20 preselected dairy farms

	Dairy cows			Young stock				Environment				Humans
Farm	MRSA/ QMS[1]	MRSA/ cows (n/n)	MRSA/ BTM[2]	MRSA/ calves MF[3] (n/n)	MRSA/ calves PW[4] (n/n)	MRSA/ prefresh heifers NSL[5] (n/n)	MRSA/ prefresh heifers UC[6] (n/n)	MRSA/ dust (dairy barn)[2]	MRSA/ dust (pig barn)[2]	MRSA in teat liners[2]	MRSA in calf feeders[2]	MRSA in humans NSL[2]
1	4/120	1/30	+	1/10	1/10	0/4	0/4	−	na	+	na	na
2	0/118	0/30	−	0/10	0/8	0/7	0/7	−	na	−	−	na
3	0/118	0/30	−	0/11	0/10	0/10	0/10	−	na	−	na	na
4	0/107	0/27	−	0/11	0/7	0/10	0/10	−	na	−	na	na
5	0/122	0/31	−	3/10	2/10	0/10	0/10	−	na	−	+	na
6	5/116	4/30	+	7/11	2/10	1/10	4/10	+	na	+	+	na
7	9/106	4/27	+	1/10	0/7	1/10	0/10	−	+	+	na	na
8	2/116	1/30	+	0/10	0/10	0/10	0/10	−	na	−	na	na
9	0/120	0/30	−	7/10	0/10	2/10	2/10	+	+	−	−	na
10	4/118	3/30	+	0/10	0/10	0/10	0/10	−	na	−	−	na
11	1/120	1/30	+	4/9	4/5	6/10	1/9	+	na	−	−	na
12	2/123	1/31	na	4/10	0/10	0/10	0/10	+	na	−	na	na
13	0/118	0/30	+	3/11	0/10	0/10	0/10	−	−	−	−	na
14	2/121	2/31	+	0/10	1/10	0/10	0/10	−	−	+	na	3/4
15	6/115	6/30	−	6/10	1/10	2/10	4/10	−	na	−	−	1/2
16	20/117	13/30	+	4/10	2/10	3/10	0/10	+	−	−	−	1/2
17	0/118	0/30	−	4/10	3/10	0/10	0/10	−	na	−	na	0/1
18	3/116	3/30	+	2/10	0/10	0/10	0/10	−	na	−	na	0/2
19	0/119	0/30	+	0/10	0/10	1/10	nd[5]	−	na	−	na	0/1
20	9/119	8/30	+	0/10	1/10	1/10	nd[5]	−	na	+	na	1/2
Total	67/2,347 (2.9%)	47/597 (7.9%)	12/19 (63.2%)	46/203 (22.7%)	17/187 (9.1%)	17/191 (8.9%)	11/170 (6.5%)	5/20 (25.0%)	2/5 (40.0%)	5/20 (25.0%)	2/9 (22.2%)	6/14 (42.9%)

[1]QMS = quarter milk sample

[2]Where − = negative; + = positive; na = not available.

[3]MF = milk fed.

[4]PW = postweaning.

[5]NSL = nasal.

[6]UC = udder cleft.

Table 2. Methicillin-resistant *Staphylococcus aureus* (MRSA) detection rate in quarter milk samples (QMS) and association with quarter position, cow group (primiparous, multiparous, high-risk group), somatic cell count, and farm number

Item	Category	% MRSA-positive QMS (n = MRSA/n = all QMS)	Odds ratio	95% CI of odds ratio		*P*-value
				Lower	Upper	
Fixed variables						
Group cows	Primiparous	1.7 (11/655)	Referent			
	Multiparous	2.1 (23/1,083)	1.370	0.617	3.043	0.440
	High-risk group[1]	5.5 (33/605)	2.933	1.369	6.285	0.006
Somatic cell count	Low[2]	1.3 (22/1,663)	Referent			
	High[2]	6.6 (45/680)	6.153	3.459	10.944	0.000
Quarter position	Right hind	2.7 (16/588)	Referent			
	Left hind	2.7 (16/588)	0.984	0.466	2.078	0.966
	Right front	3.1 (18/584)	1.339	0.645	2.780	0.433
	Left front	2.9 (17/587)	1.061	0.504	2.235	0.875
Random variable	**Category**	**% MRSA-positive farms[3] (n = MRSA/n = all farms)**	**Variance (SE)**	**95% CI of variance**		***P*-value**
				Lower	Upper	
Farm	Farm number	60 (12/20)	2.106 (0.939)	0.879	5.045	0.025

[1]Cows with previous *S. aureus* or MRSA report or somatic cell count in milk.
[2]Cut-off: 150,000 cells/mL in QMS from primiparous cows and 250,000 cells/mL in QMS from multiparous cows.
[3]MRSA in QMS.

3 milk recordings was 461,000 cells/mL on 1 MRSA-affected farm.

MRSA in Young Stock

The highest MRSA-positive test rate of 22.7% (46/203; 95% CI: 17.1–29.0%) was detected in nasal swabs from milk-fed calves (Table 1). In nasal swabs from postweaning calves, MRSA-positive test rate was 9.1% (17/187; 95% CI: 5.4–14.2%). From prefresh heifers, both nasal and udder cleft swabs were collected. Nasal swabs were positive in 17 of 191 samples (8.9%; 17/191; 95% CI: 5.3–13.9%) and udder cleft swabs in 11 of 170 samples (6.5%; 11/170; 95% CI: 3.3–11.3%). In 13.0% (26/200; 95% CI: 8.7–18.5%) of all prefresh heifers, 1 or both samples (nasal or udder cleft swabs)

Table 3. Somatic cell counts (cells/mL) from bulk-tank milk (BTM) samples on 20 preselected dairy farms

Farm	Number of cows	MRSA in milk[1]	BTM somatic cell count			
			Second previous month	Previous month	Current month	Geometric mean last 3 mo
1	400	+	255,000	253,000	255,000	254,000
2	63	−	189,000	187,000	250,000	207,000
3	74	−	nr[2]	nr	nr	nr
4	26	−	271,000	212,000	122,000	191,000
5	883	−	241,000	121,000	132,000	157,000
6	94	+	170,000	171,000	173,000	171,000
7	27	+	402,000	206,000	440,000	332,000
8	102	+	235,000	129,000	158,000	169,000
9	180	−	135,000	248,000	81,000	139,000
10	650	+	294,000	233,000	280,000	268,000
11	122	+	nr	nr	nr	nr
12	230	+	284,000	256,000	261,000	267,000
13	126	+	176,000	151,000	223,000	181,000
14	350	+	491,000	540,000	370,000	461,000
15	700	+	271,000	310,000	287,000	289,000
16	970	+	252,000	252,000	275,000	259,000
17	412	−	237,000	256,000	412,000	292,000
18	240	+	nr	nr	nr	nr
19	240	+	295,000	227,000	452,000	312,000
20	280	+	165,000	370,000	396,000	262,000

[1]Bulk-tank milk or quarter milk samples or both.
[2]nr = not reported.

carried MRSA. From all MRSA-positive prefresh heifers (n = 26), both nasal and udder cleft swabs were collected. Regarding the 2 sampling regions, 15 of 26 (57.7%) prefresh heifers were tested positive in nasal swabs and 9 of 26 (34.6%) in udder cleft swabs. Two prefresh heifers (7.7%) carried MRSA in their nasal cavities and in their udder clefts.

According to the questionnaire, 16 of 20 farms were feeding waste milk to calves. Waste milk was defined as nonsaleable according to the prescribed withdrawal periods. On farms with MRSA detection in nasal swab samples from calves, 10 of 14 farms were feeding waste milk. On 2 MRSA-positive farms, waste milk was heat treated before feeding. Farmers from 7 farms reported that they had purchased replacement heifers in the previous 6 mo.

Detection of MRSA in Pigs, Humans, and Environmental Samples

Detection of MRSA was positive in dust samples from 5 of 20 dairy barns included in our study (25%; Table 1). On these farms, MRSA was detected in multiple other samples as well. Suckers from automatic calf feeders were sampled on 10 farms, and 2 swab samples were positive. Buckets and milk-bars for calf feeding were not sampled. In swab samples from teat liners, MRSA was detected on 5 of 20 farms (25%). Five farms in this study kept both cattle and pigs. There was MRSA detected in dust samples from 2 pig barns, while samples from the remaining 3 farms were negative. In nasal swabs from farm personnel, MRSA was detected in 6 of 14 samples (42.9%) that were obtained from 4 of 7 farms (Table1).

Molecular Typing of MRSA Isolates

All MRSA isolates that were detected and characterized in our study (n = 237) belonged to the LA-MRSA ST398 and carried the *mecA* gene. No isolate carried the *pvl* gene, and no isolate was tested for the *mecC* gene because all MRSA isolates carried the *mecA* gene. Most isolates were characterized as SCC*mec*-type V and *spa*-types t011 and t034 (Table 4). Rarely detected *spa*-types were t1403, t571, and t2011. Additionally, MRSA from BTM and QMS from 2 farms, which were located in the same village, carried the unusual *spa*-type t1928. Both farms were clients of the same veterinary practice. On 13 farms, MRSA from milk samples (QMS or BTM) harbored the same *spa*-type as MRSA in samples from young stock or the environment (Table 4). There was MRSA detected with different *spa*-types in BTM and young stock on farms no. 13 and 19. On 2 more farms (no. 16 and 20), MRSA with 3 different *spa*-types were detected in QMS from the same farm. In 4 herds (no. 6, 11, 15, and 16), MRSA with identical *spa*-types were detected in all young stock populations within the same farms (milk-fed calves, postweaning calves, and heifers; Table 4). On 1 of 2 farms with MRSA-positive dust samples from pig barns, MRSA with the same *spa*-type (t011) was detected in the pig and the dairy barns. On the other farm, MRSA with different SCC*mec*- and *spa*-types were found in the pig barn (SCC*mec*-type V and *spa*-type t1451, respectively) and in QMS (SCC*mec*-type IVa and *spa*-type t011, respectively). One heifer that was kept in the same barn as the pigs carried the pig strain in her nasal cavities. The MRSA *spa*-types of all human isolates were also detected in MRSA from cattle on the corresponding farms (Table 4).

Milking-Time Hygiene

Milking-time hygiene procedures on the 20 preselected study farms are presented in Table 5. Milkers were not using gloves on 2 MRSA-affected farms. On 3 farms, some milkers were using gloves and some were not. In 2 MRSA-affected herds, no udder cleaning was performed. Five farms did not implement any cluster disinfection, and on 1 farm we observed that the automatic system for cluster disinfection was not working on several milking units. On 2 MRSA-affected farms, no postdipping was performed, and multiple teats per cow were not covered with dipping solution on 3 more farms. Finally, on 8 farms, cows that suffered from mastitis were not separated and milked last.

On farm no. 7, a smallholder tiestall barn, in which 4 of 27 cows carried MRSA, 1 udder towel was used for all cows, and MRSA was detected in teat liners (Tables 1 and 5). Moreover, no cluster disinfection and postdipping was performed. Farm no. 16, which had the highest proportion of MRSA-positive cows (43%; 13/30), recently moved to a robotic milking system. Although it was a large herd (970 cows) with high *S. aureus* detection rates from mastitis samples during the last year, infected cows were not separated from the herd. Additionally, postdipping was not working properly on several robotic milking units.

DISCUSSION

This is the first study that systematically screened samples from different groups of cattle, pigs, humans, and the environment for the occurrence of MRSA on more than 3 dairy farms. All MRSA isolates from different farms and different samples in our study belonged to LA-MRSA ST398. Previous studies from Europe reported ST398 to be the predominant MRSA strain in dairy herds (Vanderhaeghen et al., 2010; Fessler et al.,

2012; Cortimiglia et al., 2016; Tenhagen et al., 2018; Hansen et al., 2019). In Italy, both ST398 and ST97 seem to be dominant MRSA strains in dairy herds (Luini et al., 2015; Feltrin et al., 2016; Locatelli et al., 2017). The MRSA-positive test rate in our study is difficult to compare because study farms were selected based on previous MRSA reports. The MRSA-positive test rate in dairy cows from high-risk farms in this study was 7.9%. A German mastitis laboratory recently screened all milk samples they obtained in March 2017 (n = 14,924) for the presence of LA-MRSA ST398. The authors reported 10 LA-MRSA among 372 *S. aureus* isolates, concluding that LA-MRSA is not a major mastitis pathogen (Kadlec et al., 2019). A study that tested 173 *S. aureus* from intramammary infections reported 5 MRSA isolates from 2 German dairy farms (Bolte et al., 2020). In Belgium, LA-MRSA were detected on 9.3% (11/118) of dairy farms (Vanderhaeghen et al., 2010). In studies that tested BTM in Europe, MRSA was detected in 3 to 10% of samples (Cortimiglia et al., 2016; Tenhagen et al., 2018; Hansen et al., 2019). On 5 farms from our study, more than 10% of preselected cows carried MRSA. The highest percentage of positive cows was 43%, indicating that LA-MRSA may be widespread in individual herds. A high within-herd prevalence of LA-MRSA ST398 was reported from individual farms in Italy (60%, n = 33/55 and 23%, 14/59) and Israel (13%, n = 139/1050; Locatelli et al., 2017; Falk, 2018; Barberio et al., 2019). Due to resistance against β-lactam antibiotics in MRSA, segregation and culling of infected animals has been recommended to remove MRSA infected cows from the herds (Spohr et al., 2011). Lactational therapy with non-β-lactam antibiotics (e.g., pirlimycin) or therapeutic use of bacteriophages may be investigated as therapeutic options in the future (Skoulikas et al., 2018; Titze et al., 2020). Because treatment of chronic MSSA or MRSA infections is generally not recommended, these treatments can only be an option in individual cases (e.g., newly infected primiparous cows).

In our study, MRSA with similar *spa*-types were isolated from milk samples and other sample types (e.g.,

Table 4. Molecular typing results from methicillin-resistant *Staphylococcus aureus* (MRSA) strains detected in different samples from 20 preselected dairy farms

Farm (no.)	MRSA-positive sample	Sequence type	SCC*mec* types	*spa*-types
1	QMS,[1] calves, teat liners	398	V	t011
	QMS	398	V	t1451
5	Calves, calf feeder	398	V	t034
6	QMS, BTM,[2] calves, heifers, dust, teat liners, calf feeder	398	V	t034
7	QMS, BTM, calves, teat liners	398	IVa	t011
	Pigs, heifer	398	V	t1451
8	QMS, BTM	398	V	t034
9	Calves, heifers, pigs	398	V	t011
10	QMS	398	V/nd[3]	t1403
	BTM	398	V	t034
11	QMS, BTM, calves, heifers, dust	398	V	t034/nd
12	QMS, calves, dust	398	V	t011/nd
13	Calves	398	V	t571
	BTM	398	V	t011
14	QMS	398	V	t2011
	BTM, calves	398	V	t011
	Humans, teat liners	398	V	t2011/t011
15	QMS, calves, heifers, humans	398	V	t034
16	QMS	398	V	t011 (n = 4)/t034 (n = 14)/ t571 (n = 1)
	BTM, calves, heifers, dust, humans	398	V	t034
17	Calves	398	V	t034
18	QMS, BTM	398	V	t011
	Calves	398	V	t011/t034
19	Heifers	398	V	t034
	BTM	398	V	t1928
20	QMS	398	V	t011 (n = 5)/t034 (n = 1)/ t1928 (n = 3)
	BTM, calves	398	V	t011
	Teat liners	398	V	t011/t034
	Heifers, humans	398	V	t034

[1]QMS = quarter milk samples.

[2]BTM = bulk-tank milk.

[3]nd = not detected.

Table 5. Detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in milk samples and milking-time hygiene procedures on 20 preselected dairy farms

Farm (no.)	MRSA in milk[1]	Milkers use gloves	Udder cleaning	One towel per cow	Cluster disinfection	Predipping and spraying	Postdipping and spraying (%)	Mastitis group (milked last)
1	Yes	Yes	PT[2]	Yes	Sporadically	No	90–100	Yes
2	No	AMS[3]	AMS (wash)	AMS	Yes	No	90–100	No
3	No	AMS	AMS (wash)	AMS	No	No	50	No
4	No	No	CT[4]	Yes	Yes	No	50	Yes
5	No	Yes	PT	Yes	Yes	No	90–100	Yes
6	Yes	Sporadically	No	No	Yes	No	90–100	No
7	Yes	No	CT	No	No	No	No	No
8	Yes	No	PT	Yes	No	No	90–100	No
9	No	Yes	CT	Yes	Sporadically	Yes	90–100	No
10	Yes	AMS	AMS (brush)	AMS	Yes	No	90–100	Yes
11	Yes	Yes	No (parlor)	No	No	No	90–100	No
		AMS	AMS (brush)	AMS	(AMS and parlor)			
12	Yes	AMS	AMS (wash)	AMS	No	No	90–100	No
13	Yes	AMS	AMS (brush)	AMS	Yes	No	90–100	Yes
14	Yes	Sporadically	PT	Yes	Yes	No	90–100	Yes
15	Yes	Sporadically	PT	Yes	No	No	50	Yes
16	Yes	AMS	AMS (wash)	AMS	Yes	No	50	No
17	No	Yes	CT	Yes	Yes	No	No	Yes
18	Yes	AMS	AMS (wash)	AMS	Yes	No	No	No
19	Yes	Yes	WS[5]	Yes	Yes	No	90–100	No
20	Yes	Yes	PT	Yes	Yes	No	50	Yes

[1]Quarter milk sample or bulk-tank milk or both.

[2]PT = paper towel.

[3]AMS = automatic milking system.

[4]CT = cotton towel.

[5]WS = wood shavings.

young stock, environment, and humans) from most MRSA-affected herds (13/17). This indicated a spillover of LA-MRSA strains between dairy cows, young stock, or the environment on these farms. Detection of MRSA with an unusual *spa*-type (t1928) from 2 farms that were located in the same village may indicate MRSA transmission via people visiting both farms, other living vectors (e.g., flies or rodents), and dust (wind). In our study, different MRSA *spa*-types were detected in BTM and in samples from young stock on some farms, while multiple different *spa*-types were detected in QMS of other farms. The different MRSA ST398 subtypes were probably introduced via different routes (e.g., humans, replacement animals, and environmental vectors) into the respective farms. Another explanation for different *spa*-types could be a genetic recombination process such as mutations or duplications because some *spa*-types (e.g., t011 and t2011) differ by only one 16-base pair repeat (Santos-Júnior et al., 2016). Similar LA-MRSA genotypes in humans and cattle on 4 farms confirmed the potential contagious spread of LA-MRSA ST398 on German dairy farms. Previous studies from Europe reported the occurrence of LA-MRSA ST398 in samples from humans who had direct contact with cattle (Graveland et al., 2010; Fessler et al., 2012; Locatelli et al., 2017). Employees on dairy farms should be aware of possible zoonotic and reverse-zoonotic MRSA transmission, especially during the milking process, but also in relation to calf feeding.

In our study, the geometric mean somatic cell count in QMS that carried MRSA (345,000 cells/mL) was higher compared with all QMS (114,000 cells/mL), indicating that LA-MRSA ST398 caused an inflammatory response in the cows' udders. In 32.8% (22/67) of MRSA-carrying QMS, somatic cell counts were low (<150,000 cells/mL in primiparous cows and <250,000 cells/mL in multiparous cows). Somatic cell counts may vary between milkings in MSSA-affected cows, and longitudinal studies on MRSA-affected cows previously reported high variations in somatic cell counts (Pilla et al., 2012; Magro et al., 2018). Elevated somatic cell counts (>250,000 cells/mL) in BTM from the last 3 monthly milk recordings were found in the majority of MRSA-affected herds in this study and are common indicators for the presence of contagious mastitis-causing pathogens (Barkema et al., 2006; Keefe, 2012). Because our study was focused solely on MRSA, the occurrence of other mastitis pathogens as a cause of elevated BTM somatic cell count was not investigated and remains unclear.

No differences in parity (primiparous vs. multiparous) or affected mammary quarter position on the udder were observed among the 57 infected dairy cows. Previous studies on *S. aureus* risk factors reported that hind quarters were more often affected and that older cows were more likely to suffer from mastitis caused by *S. reus* (Deluyker et al., 2005; Barkema et al., 2006).

The MRSA-positive test rate in nasal swab samples from milk-fed calves was high in this study (22.7%), indicating that young calves could act as a LA-MRSA reservoir on dairy farms. In the Netherlands, LA-MRSA was detected in nasal swabs from calves on 6 of 24 farms, which is lower in comparison to our study (14/20 farms) in which high-risk farms were selected (Fessler et al., 2012). A high positive test rate for LA-MRSA ST398 of up to 82% was reported from veal calves in Europe (Bos et al., 2012; Vandendriessche et al., 2013; Tenhagen et al., 2014). However, housing and production systems in veal calf farms differ significantly from calf rearing systems on dairy farms. Veal calf farms usually raise animals from multiple different facilities, increasing the risk for MRSA introduction via calves and cattle traders. In our study, all calves were born on the individual farms. Because most dairy farms in this study practice waste milk feeding, contaminated milk might introduce MRSA into the calf population (Ricci et al., 2017). A German study reported the presence of MRSA-positive nasal swabs from calves fed MRSA-contaminated milk (Spohr et al., 2011). Additionally, some MRSA-positive QMS showed low somatic cell counts. Therefore, MRSA-contaminated raw milk from presumably healthy cows might be fed to calves and enter the dairy food chain. Other causes of MRSA spillover to calves might be from contact with farm personnel, dust, colostrum feeding, or direct contact with cows during or after parturition. Within groups of calves, MRSA might be transmitted through direct contact and suckers from automatic calf feeders, as detected in this study. The MRSA-positive test rate in nasal swabs from postweaning calves (9.1%) and prefresh heifers (8.9%) was lower than in nasal swabs from milk-fed calves. Major changes in calf immunity and in nasal microbiota composition might lead to competitive exclusion of LA-MRSA with increasing age (Chase et al., 2008; Holman et al., 2015). Additionally, frequent MRSA exposure and the consequent risk for reinfection via milk feeding and suckers is not given in postweaning cattle. For MRSA with identical *spa*-types in swab samples from milk-fed calves, postweaning calves and prefresh heifers from 4 farms showed that MRSA strains may persist in the nasal cavities of young stock with increasing age. Although MRSA-positive test rate was lower in prefresh heifers compared with calves, MRSA detection in nasal and udder cleft swabs showed that replacement heifers may introduce MRSA into dairy herds. Regarding the proportions of MRSA-positive samples from nasal swabs (57.7%; 15/26) and udder cleft swabs (34.6%; 9/26) in prefresh heifers, it can be

concluded that detection rates were low in both sample types. Only 2 prefresh heifers tested positive in both sample types. Therefore, if farmers intend to check the MRSA status of replacement heifers, samples from multiple body sites should be included in parallel testing.

Although results from previous studies on milking-time hygiene were not always consistent, proper milking-time hygiene procedures to prevent the spread of *S. aureus* should include the following procedures: (1) use of clean ves during milking, (2) application of a postmilking teat disinfectant, (3) milking infected cows last, and (4) use of 1 cloth per cow for drying and cleaning teats (Keefe, 2012; Edmondson, 2020; Graber, 2020). The use of a predipping solution was also shown to reduce the spread of *S. aureus* (Dufour et al., 2012). The effect of cluster disinfection and cleaning on the spread of contagious mastitis pathogens was not always consistent, and in some countries, cluster disinfection was not allowed (Keefe, 2012; Edmondson, 2020). According to the German Institute for milk testing, cluster disinfection is a recommended procedure in *S. aureus* control programs (IfM GmbH and Co. KG Institut für Milchuntersuchung, Verden, Germany). Results from milking hygiene evaluation in our study showed that MRSA-affected study farms were not consistently following milking-time hygiene guidelines to prevent contagious mastitis. Studies from Brazil and Italy also reported improper milking hygiene procedures on MRSA-affected farms (Antoci et al., 2013; Guimarães et al., 2017; Locatelli et al., 2017). In a case report from Israel about a severe LA-MRSA ST398 outbreak in 2018 to 2019 (Falk, 2018), the authors reported a change of milking parlor and milking procedures in 2017 on the affected farm (personal communications). Similarly, in our study, the farm with the highest proportion of MRSA-positive cows (43%) recently moved to a new robotic milking system; postdipping was not working on several milking units and MRSA-affected cows were not separated from the herd. The results underline the need for proper milking-time hygiene and technique to prevent the spread of MRSA within dairy herds, just as for any other contagious *S. aureus* case.

In this study, the same MRSA genotype was detected on only 1 of 5 farms that kept both cattle and pigs. On another farm, different SCC*mec* and *spa*-types were detected in the pig environment, indicating no current MRSA spillover from pigs to dairy cows or vice versa. Several studies reported higher LA-MRSA in dairy herds from areas with high pig density, and possible transmission between the species (Spohr et al., 2011; Tavakol et al., 2012; Locatelli et al., 2016). A recent Danish study reported high genetic relatedness of LA-MRSA ST398 genotypes from cattle and pigs (Hansen et al., 2019). In the Netherlands, LA-MRSA genotypes from cattle and pigs were the same on some farms, but differed on other farms (Fessler et al., 2012). An Italian study did not find a correlation between MRSA status and the presence of other animal species, including pigs, on MRSA-affected farms (Cortimiglia et al., 2016). Therefore, further research is needed to investigate the role of pigs as a potential source of LA-MRSA infections in dairy cows.

For future MRSA monitoring on dairy farms we recommend combining BTM samples and nasal swab samples of milk-fed calves because MRSA detection rates were the highest in these samples. Taking both sample types into consideration, all MRSA-positive farms (17/17) would have been identified in our study.

CONCLUSIONS

Detection of LA-MRSA ST398 was found on 17 of 20 preselected dairy farms; it spreads among different groups of animals, humans, and in the environment. Milk-fed calves in particular, but also postweaning calves and heifers may be a reservoir of LA-MRSA on dairy farms. No difference in MRSA-positive test rate was observed between primiparous versus multiparous cows and quarters. High-risk cows and QMS with high somatic cell counts were more likely to carry MRSA. Improper milking-time hygiene procedures and elevated BTM somatic cell counts were common features of MRSA-affected farms in this study, as it is known for MSSA. Detection of MRSA in farm personnel confirms the high probability of zoonotic and reverse-zoonotic LA-MRSA transmission on dairy farms. Frequent spillover of LA-MRSA ST398 from pigs to dairy cattle could not be confirmed in our study because similar genotypes were detected on only 1 farm, and MRSA *spa*-types from the pig barn and dairy cows were different on 1 more farm. High MRSA detection rates in BTM and nasal swab samples of milk-fed calves indicate that these sample types could be used for MRSA-monitoring programs in dairy herds.

ACKNOWLEDGMENTS

This study was carried out in the framework of the project #1HealthPREVENT, funded by the German Federal Ministry of Education and Research, grant no. 01KI1727C. We thank all farmers who voluntarily participated in the study. Thanks to Ulrike Sorge, Thomas Peters, Karsten Donat, Sabine Reinhold, Oliver Claushues, Karin Eulenberger, and Natalie Morgenstern for supporting the identification of study farms. Thanks to Stefan Borchardt for support with herd management

software. Thanks to Roswitha Merle for support with statistical analysis. The authors have not stated any conflicts of interest.

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3.3 Publication 3

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The occurrence of methicillin resistant non-*aureus* staphylococci in samples from cows, young stock and the environment of German dairy farms

https://doi.org/10.3168/jds.2020-19704

Journal of Dairy Science

April 2021; 104(4);4604-4614.

J. Dairy Sci. 104:4604–46
https://doi.org/10.3168/jds.2020-19704

The occurrence of methicillin-resistant non-*aureus* staphylococci in samples from cows, young stock, and the environment on German dairy farms

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ABSTRACT

This study aimed to determine the occurrence of methicillin-resistant (MR) non-*aureus* staphylococci (NAS) on 20 preselected German dairy farms. Farms were selected based on the detection of methicillin-resistant *Staphylococcus aureus* (MRSA) during previous diagnostic investigations. Bacterial culture of presumptive MR-NAS was based on a 2-step enrichment method that has been recommended for MRSA detection. Quarter milk samples (QMS), bulk tank milk, swab samples from young stock, and environmental samples were collected for bacterial culture. Methicillin-resistant NAS were detected on all study farms. The MR-NAS positive test rate was 3.3% (77/2,347) in QMS, 42.1% (8/19) in bulk tank milk, 29.1% (59/203) in nasal swabs from milk-fed calves, 18.3% (35/191) in postweaning calves, and 7.3% (14/191) in nasal swabs from prefresh heifers. In the environment, MR-NAS were detected in dust samples on 25% (5/20) of the dairy farms as well as in teat liners and suckers from automatic calf feeders. The geometric mean somatic cell count in QMS affected by MR-NAS (183,000 cells/mL) was slightly higher compared with all QMS (114,000 cells/mL). Nine MR-NAS species were identified; *Staph. sciuri*, *Staph. lentus*, *Staph. fleurettii*, *Staph. epidermidis*, and *Staph. haemolyticus* were the most common species. In addition, 170 NAS isolates were identified that showed reduced cefoxitin susceptibility (4 mg/L) but did not harbor the *mecA* or *mecC* genes. On some farms, similar mobile genetic elements were detected in MR-NAS and MRSA. It was suggested that resistance genes may be transferred between NAS and *Staph. aureus* on the respective farms.

Key words: methicillin, non-*aureus* staphylococci, coagulase-negative staphylococci, antimicrobial resistance, dairy cattle

Received September 28, 2020.
Accepted December 8, 2020.
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INTRODUCTION

Non-*aureus* staphylococci are a diverse group of bacteria that have been detected on dairy farms worldwide. Because most NAS species are coagulase negative, this group of bacteria was formerly referred to as "coagulase-negative staphylococci." In different studies about 8% of mastitis milk samples carried NAS (Ruegg, 2018). Although NAS have been regarded as minor mastitis-causing pathogens, the effect of NAS on udder health and milk quality was reported to be higher in heifers and in herds with overall good udder health (Schukken et al., 2009; Condas et al., 2017). The most frequently detected NAS species from mastitis milk samples of dairy cows are *Staph. chromogenes*, *Staph. simulans*, *Staph. xylosus*, *Staph. haemolyticus*, and *Staph. epidermidis* (Vanderhaeghen et al., 2015; Condas et al., 2017). Studies on epidemiology, host adaption, and pathogenicity of NAS provide evidence that NAS species can act as commensals, opportunistic and obligate pathogenic bacteria (Supré et al., 2011; De Visscher et al., 2014). *Staphylococcus chromogenes* especially seems to be host adapted and pathogenic, and other NAS species (e.g., *Staph. haemolyticus*, *Staph. fleurettii*, and *Staph. equorum*) should be regarded as opportunistic environmental pathogens (Vanderhaeghen et al., 2015; De Visscher et al., 2017; Jenkins et al., 2019).

Methicillin-resistant (**MR**) NAS are resistant to β-lactam antibiotics, which is the most important group of antibiotics approved for mastitis therapy in dairy cows. Broad-spectrum β-lactam resistance in staphylococci is often mediated by the *mecA* or *mecC* genes, which encode for the modified penicillin binding protein 2a and are well known from MR *Staph. aureus* (**MRSA**). The diversity of *mecA* genes is higher in NAS and MR-NAS compared with *Staph. aureus* and MRSA (Becker et al., 2014; Miragaia, 2018). In addition, MIC for phenotypic methicillin resistance testing using cefoxitin or oxacillin are highly heterogeneous (Dickinson and Archer, 2000). Therefore, the overall burden of MR-NAS is difficult to evaluate, and comparison between studies is challenging due to different sample types and detection methods.

The most frequently detected MR-NAS species from mastitis in dairy cows are MR *Staph. epidermidis* (**MRSE**; Gindonis et al., 2013; Seixas et al., 2014; Nobrega et al., 2018) and MR *Staph. sciuri* (Cicconi-Hogan et al., 2014; Mahato et al., 2017; Fisher and Paterson, 2020). In hospitals, MRSE emerges as a major pathogen associated with immunocompromised patients and foreign body infections (Becker et al., 2020).

Numerous studies have reported methicillin resistance in NAS isolates from mastitis milk samples; however, little is known about the occurrence and distribution of MR-NAS in the different habitats on dairy cattle farms. Therefore, we aimed to determine the occurrence and genotypic characteristics of MR-NAS isolates from different age groups of cattle and environmental samples on preselected German dairy farms that had a history of MRSA detection.

MATERIALS AND MEHODS

Sampling

Twenty dairy herds from different regions in Germany were included in our study. Selection process and herd characteristics of the study farms were previously described (Schnitt et al., 2020). All herds were selected based on previous findings of oxacillin-resistant *Staph. aureus* isolates that were identified by mastitis laboratories. Because all samples were collected in the framework of a diagnostic investigation, no ethical approval was required according to the German legislation. Quarter milk samples (**QMS**) were collected aseptically by a trained veterinarian according to the guidelines of the German veterinary association (DVG, 2009). Teats were dry cleaned with a single-use paper towel and 3 streams were stripped in a milking cup. Teat ends were disinfected with 70% ethanol solution for approximately 15 s, and 1 to 3 streams of milk were collected in a sterile tube (TPP AG, Trasadingen, Switzerland).

In total, 3,167 samples were collected for bacterial culture; 2,347 QMS from 597 dairy cows were included in our study, and bulk tank milk (**BTM**) was obtained from 19 farms. On each farm approximately 30 dairy cows were sampled. In detail, 10 high-risk cows were selected based on previous MRSA reports or current high SCC in milk. Additionally, 10 primiparous and 10 multiparous cows were randomly selected during the milking process on each farm. Nasal swabs were collected from milk-fed calves (n = 203), postweaning calves (n = 187), and prefresh heifers (n = 191). Swab samples were additionally collected from the udder cleft of prefresh heifers (n = 170). On each farm, approximately 10 milk-fed calves, 10 postweaning calves, and 10 prefresh heifers were randomly selected. On each farm, dust samples (n = 20) were collected by wiping barn surfaces such as walls and cubicle tubes with a boot swab. In addition, swab samples from teat liners (n = 20) were collected. A swab sample from the suckers of automatic calf feeders was collected on 9 farms.

Isolation and Molecular Characterization of MR-NAS

Screening for MR staphylococci was performed using a double selective enrichment method, which was developed for MRSA detection (EFSA, 2007; Nemeghaire et al., 2014). The double selective enrichment method has been used for MRSA isolation from cattle before and is recommended by the European Food Safety Authority (EFSA, 2012; Nemeghaire et al., 2014). Samples were incubated in Mueller Hinton (**MH**) broth supplemented with 6.0% of NaCl for 24 ± 2 h followed by a transfer of 1 mL of MH broth in 9 mL of tryptic soy broth supplemented with 3.5 mg/L cefoxitin and 50 mg/L aztreonam and incubation at 37°C for 24 ± 2 h. After an ternal validation process at the German National Reference Laboratory for Coagulase Positive Staphylococci, the salt concentration of the usually recommended MH broth was slightly reduced from 6.5% to 6.0% and the aztreonam content of the tryptic soy broth was reduced from 75 mg/L to 50 mg/L (EFSA, 2007; Tenhagen et al., 2014). The enrichment broth (50 μL) was streaked on mannitol salt agar (**MSA**) containing 4 mg/L cefoxitin and incubated for 24 ± 2 h at 37°C. Each BTM sample was incubated in 3 batches of 1 mL of BTM and 9 mL of MH broth with 6.0% NaCl for 48 ± 2 h followed by transfer on MSA-cefoxitin agar. All colonies from MSA-cefoxitin plates (QMS, BTM, and swab samples) were transferred on sheep blood agar plates (Oxoid GmbH, Wesel, Germany) and incubated for 24 ± 2 h. Colonies from sheep blood agar plates were further analyzed by a MALDI-TOF mass spectrometer according to the manufacturer's instructions (Bruker Scientific LLC, Billerica, MA). Colonies were directly transferred on the MALDI-TOF target as previously described (Cameron et al., 2017). Colonies were further covered with 1.0 μL of α-cyano-4-hydroxycinnamic acid (Bruker Scientific LLC). The reference database for species identification was provided by Bruker Scientific LLC (MBT-BDAL-8468). If phenotypically different colonies were observed on sheep blood agar plates, they were separately spotted on the MALDI-TOF target.

Further analysis included a PCR for detection of the *tuf* gene specific for staphylococci and the *mecA* gene (Kilic et al., 2010; Fosheim et al., 2011). For staphylococci that carried the *mecA* gene, an additional PCR for typing of the staphylococcal cassette chromosome

mec (**SCC*mec***) was performed (Zhang et al., 2005). A PCR for the detection of the *mecC* gene was performed for isolates that did not carry the *mecA* gene (García-Álvarez et al., 2011). Presumptive MR-NAS isolates that were not identified by MALDI-TOF but that carried the *tuf* gene, specific for staphylococci, were summarized as *Staphylococcus* spp. The NAS isolates that grew on the selective agar and carried the *mecA* gene were considered MR-NAS in this study. Other staphylococci growing on the selective medium but not carrying the *mecA* or *mecC* gene were named "NAS with reduced cefoxitin susceptibility" and are presented separately. Somatic cell counts in QMS were measured using a DeLaval cell counter (DeLaval International, Tumba, Sweden) according to the manufacturer's instructions.

Statistical Analysis

Positive test rate of MR-NAS and 95% confidence interval were determined (positive test rate = number of MR-NAS-positive samples/number of all samples from the specific population). The Mann-Whitney U test was used to compare the SCC in QMS affected and unaffected by MR-NAS. The SPSS multilevel binary logistic regression model was used to analyze associations between MR-NAS status and SCC, quarter position, and cow group (primiparous, multiparous, and high-risk group). Farm number was included as a hierarchical random effect. Alpha was set at 0.05. Analyses were carried out in SPSS version 26.0 (IBM Corp., Armonk, NY).

RESULTS

Detection of MR-NAS in Samples from Dairy Cows

Methicillin-resistant NAS were detected on 19/20 dairy farms included in our study (Table 1). The MR-NAS positive test rate in QMS from dairy cows was 3.3% (77/2,347; 95% CI: 2.6–4.1%). The MR-NAS positive test rate in QMS from randomly selected primiparous cows was 2.3% (15/657; 95% CI:1.3–3.7%); in QMS from randomly selected multiparous cows the positive test rate was 4.6% (50/1,083; 95% CI: 3.4–6.0%), and in the high-risk group the positive test rate was 2.2% (13/603; 95% CI: 1.2–3.7%). In 33.9% (19/56) of MR-NAS-positive cows, multiple quarters were affected. On the cow level, MR-NAS were detected in 9.4% (56/597; 95% CI: 7.2–12.0%) of the dairy cows. In BTM, 42.1% (8/19) of samples carried MR-NAS. In 1 BTM sample, 3 different MR-NAS species were detected, whereas in the remaining positive samples only 1 species was identified.

Table 1. Methicillin-resistant (MR) NAS in samples from dairy cows, young stock, and the environment on dairy farms

MR-NAS species	Dairy cows		Young stock[1]				Environment			Total (no.)
	Quarter milk samples (no.)	Bulk tank milk (no.)	Calves, MF (no.)	Calves, PW (no.)	Prefresh heifers, NSL (no.)	Prefresh heifers, UC (no.)	Dust (no.)	Teat liners (no.)	Calf feeder (no.)	
Staph. sciuri	32	1	29	8	4	4	3	3	4	88
Staph. lentus	1		18	18	6	1	1	1	1	47
Staph. fleurettii	10			2	2	2		1		17
Staph. epidermidis	5	1	4	1		2		1		1
Staph. haemolyticus	6	2	2				1			11
Staph. cohnii	6							1		7
Staph. capitis						1				1
Staph. vitulinus				1	1					2
Staph. kloosii					1					1
Staph. spp.[2]	17	4	6	5	1	1				33
Total										
No.[3]	77/2,347	8/19	59/203	35/191	14/191	12/170	5/20	7/20	5/9	222/3,167
%	3.3	42.1	29.1	18.3	7.3	7.1	25	35	55.6	7.0

[1]MF = milk-fed; PW = postweaning; NSL = nasal; UC = udder cleft.
[2]*Staphylococcus* species that were not identified by MALDI-TOF but carried the *tuf* gene specific for staphylococci.
[3]MR-NAS/all samples.

The geometric mean SCC of all QMS that carried MR-NAS was 183,000 cells/mL, and the geometric mean SCC of all QMS from our study was 114,000 cells/mL. Somatic cell counts were significantly higher in quarters affected by MR-NAS compared with all QMS ($P = 0.001$).

The association between MR-NAS status and cow group (primiparous, multiparous, and high risk), SCC, and affected quarter position is presented in Table 2. High SCC was defined as >150,000 cells/mL in primiparous cows and >250,000 cells/mL in multiparous cows. The QMS with high SCC were approximately 1.8 times more likely to carry MR-NAS compared with QMS with low SCC (odds ratio: 1.838; 95% CI: 1.119–3.019; $P = 0.019$). In addition, QMS from randomly selected multiparous cows were approximately 2 times more likely to carry MR-NAS compared with QMS from randomly selected primiparous cows (odds ratio: 1.950; 95% CI: 1.042–3.649; $P = 0.038$). No difference in MR-NAS positive test rate from QMS was observed between primiparous and high-risk cows or between the affected mammary quarter positions (right hind, left hind, right front, left front; Table 2; $P > 0.05$).

Detection of MR-NAS in Samples from Young Stock and the Environment

The MR-NAS positive test rate in nasal swab samples from milk-fed calves was 29.1% (59/203; 95% CI: 22.0–34.8%), and in postweaning calves the positive test rate was 18.3% (35/191; 95% CI: 13.4–25.1%; Table 1). In 12.5% (25/200; 95% CI: 8.3–17.9%) of all prefresh heifers, one or both samples (nasal or udder cleft swabs) carried MR-NAS. In nasal swab samples 7.3% (14/191; 95% CI: 4.1–12.0%) carried MR-NAS, and 7.1% (12/170; 95% CI: 3.7–12.0%) of udder cleft swab samples tested positive. One prefresh heifer carried MR *Staph. sciuri* in the nose and in the udder cleft. Dust samples were collected from all 20 dairy farms, and MR-NAS were detected in 25% (5/20) of the samples. In swab samples from teat liners, MR-NAS were detected on 35.0% (7/20) of the farms, and suckers from automatic calf feeders tested positive on 55.6% (5/9) of the dairy farms that used automatic feeders.

MR-NAS Species

Nine NAS species were identified by MALDI-TOF (Table 1). *Staphylococcus sciuri* was the most frequently detected MR-NAS species in this study (n = 88). *Staphylococcus sciuri* was the only species that was detected in all sample types from this study and the most frequently detected species in QMS from dairy cows (n = 32; Table 1). The second most common MR-NAS from QMS was *Staph. fleurettii* (n = 10), followed by *Staph. haemolyticus* (n = 6), *Staph. cohnii* (n = 6), and *Staph. epidermidis* (n = 5). In nasal swab samples from calves, *Staph. sciuri* (n = 37) and *Staph. lentus* (n = 36) were the most frequently detected MR-NAS species. A similar distribution was found in prefresh heifers, where *Staph. sciuri* (n = 8), *Staph. lentus* (n = 7), and *Staph. fleurettii* (n = 6) were mostly detected. In the environment MR *Staph. sciuri* were isolated from dust (n = 3), teat liners (n = 3), and suckers from automatic calf feeders (n = 4) from 9 farms. *Staphylococcus lentus* was detected in dust (n = 1), teat liners (n = 1), and from 1 automatic calf feeder. Additional MR-NAS species that were detected in up to 3 samples were MR *Staph.*

Table 2. Methicillin-resistant (MR) NAS positive test rates in quarter milk samples (QMS) and association with cow group, SCC, and quarter position

Variable	Category	MR-NAS-positive QMS (%; no.[1])	Odds ratio	95% CI of odds ratio		*P*-value
				Lower	Upper	
Cow group	Primiparous	2.3 (15/657)	Referent			
	Multiparous	4.6 (50/1,083)	1.950	1.042	3.649	0.038
	High-risk[2]	2.2 (13/603)	0.828	0.369	1.861	0.629
SCC[3]	Low	2.7(45/1,663)	Referent			
	High	4.9 (33/680)	1.838	1.119	3.019	0.019
Quarter position	Right hind	3.6 (21/588)	Referent			
	Left hind	4.6 (27/588)	1.326	0.711	2.471	0.353
	Right front	2.2 (13/584)	0.651	0.307	1.379	0.244
	Left front	2.9 (17/587)	0.840	0.419	1.683	0.603

[1]No. in parentheses = MR-NAS/all samples.

[2]Cows with previous *Staph. aureus*/MRSA report or recent high SCC in milk.

[3]Low = <150,000 cells/mL in QMS from primiparous cows and <250,000 cells/mL QMS from multiparous cows. High = >150,000 cells/mL in QMS from primiparous cows and >250,000 cells/mL QMS from multiparous cows.

vitulinus, MR *Staph. kloosii*, and MR *Staph. capitis* (Table 1).

In addition, 170 NAS isolates were detected that grew on MSA-cefoxitin agar plates (4 mg/L) but did not harbor the *mecA* or *mecC* genes (Table 3). Because the phenotypic cut-off value for cefoxitin resistance in *Staph. aureus* is 4 mg/L, these NAS isolates were considered to exhibit reduced cefoxitin susceptibility (CLSI, 2018). The most common NAS species with reduced cefoxitin susceptibility that carried neither the *mecA* gene nor the *mecC* gene was *Staph. cohnii*, which was isolated from 15/20 farms. Most *Staph. cohnii* were detected in milk samples (n = 120), and the geometric mean SCC of QMS affected by *Staph. cohnii* was 153,000 cells/mL. From calves and prefresh heifers, 12 *Staph. cohnii* isolates were obtained, and 2 *Staph. cohnii* were isolated from dust and teat liners (Table 3). Additional NAS species with reduced cefoxitin susceptibility were *Staph. pettenkoferi* (n = 3), *Staph. xylosus* (n = 2), and *Staph. saprophyticus* (n = 1) as well as 26 NAS that were not identified by MALDI-TOF but carried the *tuf* gene specific for staphylococci.

MR-NAS Detection Within Farms

The occurrence of MR-NAS species within the 20 preselected dairy farms is presented in Figure 1. The MR-NAS species that were detected fewer than 4 times in our study and isolates that were not identified by MALDI-TOF were summarized as *Staphylococcus* spp. in Figure 1. On farm 12, 17.1% (21/123) of QMS carried MR *Staph. sciuri* and the same species was detected in samples from young stock and teat liners. On farm 4, MR *Staph. sciuri* was the predominant species, especially in samples of young stock but also in QMS and dust samples. On farm 10, MR *Staph. lentus* was most frequently detected, especially from the different calf populations. In addition, high numbers of *Staph. cohnii* isolates with reduced cefoxitin susceptibility (*mecA*/*mecC* negative) were detected in QMS from farm number 1 (25.5%; 27/106) and number 7 (20.8%; 25/120). On other farms (e.g., numbers 3, 5, 11, 13, and 18), up to 5 different species were detected within farms.

Genotypic Characteristics of MR-NAS Isolates

The SCC*mec* types of most MR-NAS (86.9%; 193/222) were not identified. Eight *Staph. haemolyticus* isolates from 7 farms carried SCC*mec* type V. Additionally, 7 MR *Staph. cohnii* from 1 farm and 1 MRSE isolate from the same herd carried SCC*mec* type V. Eight MRSE isolates from 4 farms carried SCC*mec* type IVa.

Table 3. The *mecA*- and *mecC*-negative NAS species that showed reduced cefoxitin susceptibility (4 mg/L) in samples from dairy cows, young stock, and the environment on dairy farms

NAS species	Dairy cows		Young stock[1]				Environment			Total (no.)
	Quarter milk samples (no.)	Bulk tank milk (no.)	Calves, MF (no.)	Calves, PW (no.)	Prefresh heifers, NSL (no.)	Prefresh heifers, UC (no.)	Dust (no.)	Teat liners (no.)	Calf feeder (no.)	
Staph. cohnii	120	2		4	2	6	2	2		138
Staph. pettenkoferi	1			1	1					3
Staph. xylosus	2									2
Staph. saprophyticus	1		1	1	1			2		1
Staph. spp.[2]	21									26
Total										
No.[3]	145/2,347	2/19	1/203	6/187	4/191	6/170	2/20	4/20	0/9	170/3,167
%	6.2	10.5	0.5	3.2	2.1	3.5	10.0	20.0		5.4

[1]MF = milk-fed; PW = postweaning; NSL = nasal; UC = udder cleft.

[2]*Staphylococcus* species that were not identified by MALDI-TOF but carried the *tuf* gene specific for staphylococci.

[3]MR-NAS/all samples.

DISCUSSION

This is the first study that reports the occurrence of MR-NAS in QMS, BTM, swab samples from young stock, and the environment of dairy farms. The detection of MR-NAS from this study was based on a standardized laboratory panel for MRSA detection (EFSA, 2007; Nemeghaire et al., 2014). Cefoxitin-containing media were used for preselection of MR staphylococci. According to the European Committee of Antimicrobial Susceptibility Testing, phenotypic cefoxitin or oxacillin resistance has been recommended for *mecA* prediction in NAS (EUCAST, 2020). For the detection of methicillin resistance in some NAS species (*Staph. pseudintermedius* and *Staph. schleiferi*), oxacillin susceptibility was shown to be more sensitive compared with cefoxitin (Swenson and Tenover, 2005; EUCAST, 2020). *Staphylococcus pseudintermedius* was rarely detected in dairy cows and *Staph. schleiferi* was not detected in samples from cattle so far (Pilla et al., 2013). However, oxacillin and cefoxitin MIC were shown to be highly heterogeneous among NAS species. For MRSE, oxacillin MIC values between 1 and 128 mg/L were reported (Dickinson and Archer, 2000). In conclusion, NAS are a highly diverse group of bacteria, and species-specific MIC values (cefoxitin and oxacillin) are mostly unavailable and may differ from *Staph. aureus*- and MRSA-related MIC values. Consequently, some MR-NAS may have gone undetected using the cefoxitin-based selective enrichment procedure in this study. In addition, oxacillin-susceptible *mecA*-positive NAS were detected on dairy farms (Mahato et al., 2017). Oxacillin-susceptible MR-NAS would have gone undetected in this study due to the previously described enrichment method. The MR-NAS positive test rates from this study might therefore be underestimated, and comparison of MR-NAS prevalence between studies is possible only to a limited extend.

In our study, MR-NAS were detected in 3.3% (77/2,347; 95% CI: 2.6–4.1%) of the QMS from preselected dairy herds. A study from Switzerland reported 55 MR-NAS isolated from 370 QMS, which is a higher proportion compared with our results (Frey et al., 2013). However, all isolates from the Swiss study were obtained from mastitis milk samples, and the authors mentioned that from multiple samples more than 1 NAS species was isolated. Therefore, the MR-NAS prevalence was probably overestimated. Low numbers of *mecA* gene-harboring isolates among NAS isolates from different cows were detected in Canada (0.9% (4/405; Nobrega

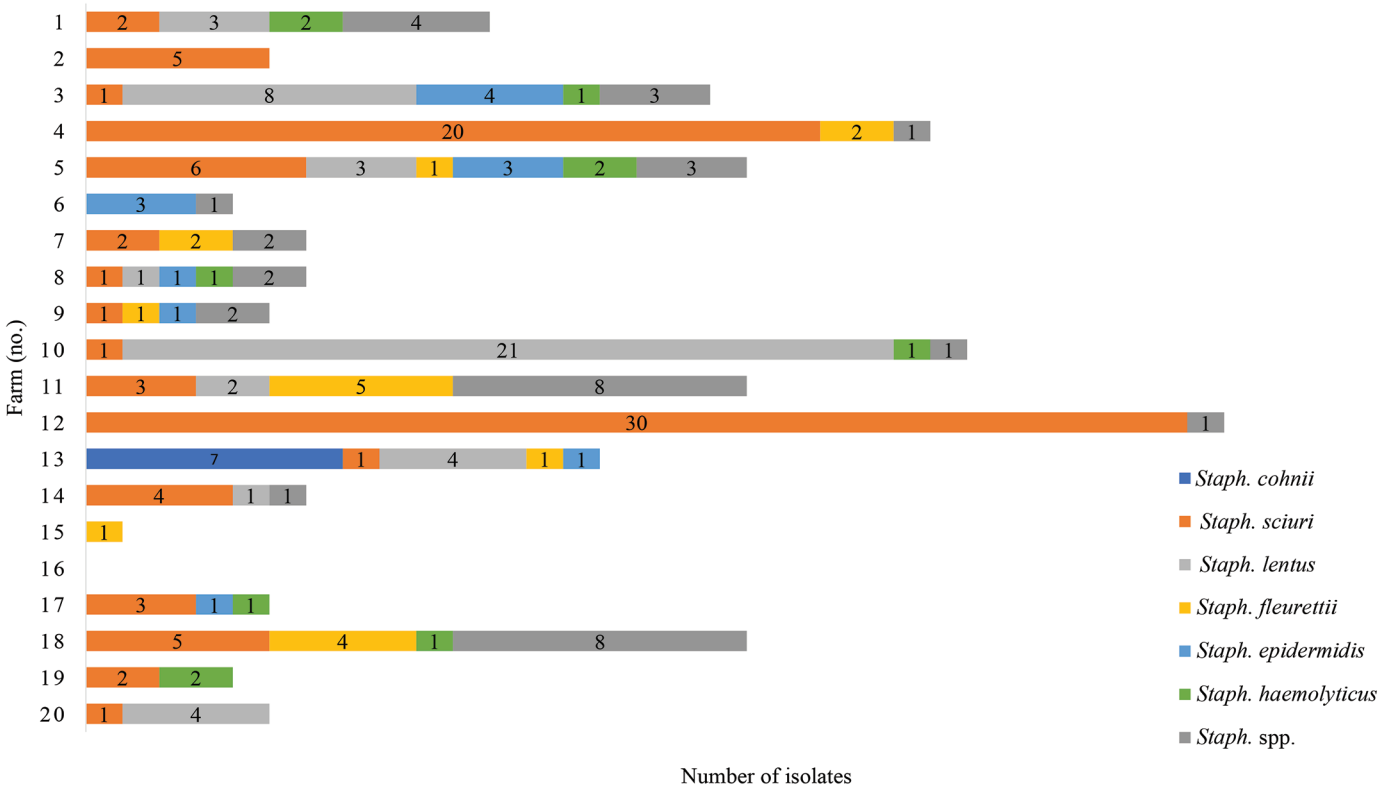

Figure 1. Methicillin-resistant NAS in samples from dairy cows, young stock, and the environment of 20 preselected dairy farms.

et al., 2018). In studies that investigated methicillin resistance of NAS from mastitis milk samples, prevalence ranged from 14.1% (26/170) in the Netherlands up to 73.2% (82/112) in a study from China (Sampimon et al., 2011; Qu et al., 2019). In our study, MR-NAS were isolated from 42.1% (8/19) of BTM samples of preselected herds. A study from Switzerland reported that 62% (62/100) of BTM samples carried MR-NAS, which is a higher proportion compared with our study (Huber et al., 2011). In the United States, 1 study reported 11 MR-NAS isolates in BTM samples from 7/288 farms (2.4%; Cicconi-Hogan et al., 2014). In the United Kingdom, 4.1% (15/363) of BTM samples carried MR-NAS (Fisher and Paterson, 2020).

In this study, randomly selected multiparous cows were more likely to carry MR-NAS in QMS compared with primiparous cows (odds ratio: 1.950; 95% CI: 1.042–3.649; $P = 0.038$). Previous studies that did not investigate methicillin resistance reported a higher NAS prevalence in primiparous cows (De Visscher et al., 2016; Condas et al., 2017). In a Canadian study, primiparous cows were 3 times more likely to carry *Staph. chromogenes* compared with multiparous cows (Condas et al., 2017). Therefore, the absence of this species in our study may explain the higher MR-NAS positive test rate in multiparous cows. Cows from the high-risk group were not more likely to carry MR-NAS than randomly selected primiparous and multiparous cows ($P = 0.629$). This can be explained by the definition of the high-risk group, which was focused on previous MRSA reports and not on MR-NAS detection. No difference in MR-NAS positive test rate between quarters (right hind, left hind, right front, left front) was observed in our study. Similarly, no significant differences in NAS prevalence between quarter positions was reported from previous studies that did not perform resistance testing (De Visscher et al., 2016; Condas et al., 2017).

The MR-NAS positive test rate among calves in our study was 29.1% (59/203; 95% CI: 22.0–34.8%) in milk-fed calves and 18.3% (35/191; 95% CI: 13.4–25.1%) in postweaning calves. A study from Belgium reported an MR-NAS carriage rate in dairy calves of 13.1%, which is lower compared with our study (Vanderhaeghen et al., 2013). In Switzerland, 62% (62/100) of nasal swabs from calves carried MR-NAS, which is a higher detection rate compared with our study (Huber et al., 2011). However, the authors of the Swiss study did not report whether dairy calves, veal calves, or beef calves were sampled.

Most studies that investigated methicillin resistance in NAS from milk samples reported MR *Staph. sciuri* (Frey et al., 2013; Mahato et al., 2017; Fisher and Paterson, 2020) and MRSE (Taponen et al., 2015; Nobrega et al., 2018; Kim et al., 2019) as the most frequently detected MR-NAS species. Methicillin-resistant *Staph. sciuri* was the most common MR-NAS species in our study as well. Methicillin-resistant *Staph. epidermidis* was detected in 5 QMS from 3 farms. In particular, MRSE was shown to exhibit low phenotypic oxacillin resistance in some cases (Mahato et al., 2017; Dickinson and Archer, 2000). Therefore, low MRSE detection rates in this study might be caused by the cefoxitin-based selection procedure in this study. The second most common MR-NAS species from QMS in our study was MR *Staph. fleurettii*, which was the most frequently detected MR-NAS species in BTM from Switzerland (Huber et al., 2011). In addition, we detected high numbers of *Staph. cohnii* (n = 120) that showed reduced cefoxitin susceptibility but did not carry the *mecA* or *mecC* gene. In previous studies, MR *Staph. cohnii* and phenotypically oxacillin-resistant *Staph. cohnii* were rarely detected (Huber et al., 2011; Frey et al., 2013). Studies that did not investigate methicillin resistance suggested that *Staph. cohnii* should be regarded as an environmental commensal NAS species on dairy farms (De Visscher et al., 2016; Wuytack et al., 2020b). In a Danish study, *Staph. cohnii* was one of the most common NAS species isolated from teat skin swabs (Mahmmod et al., 2018). We detected *Staph. cohnii* mostly in QMS, with up to 25.5% positive samples within farms. Although we performed aseptic milk sampling procedures, it cannot be excluded that some *Staph. cohnii* in QMS from our study occurred from teat-end contamination. In the field, a clean collection of QMS is sometimes difficult, especially if cows and milking parlors are dirty and time for teat cleaning is limited during the milking process. A high detection rate of *Staph. cohnii* isolates with reduced cefoxitin susceptibility in QMS and a slightly higher geometric mean SCC in *Staph. cohnii* affected quarters compared with the cell count of all QMS from this study (153,000 cells/mL vs. 114,000 cells/mL) indicate that *Staph. cohnii* could act as a cow-associated opportunistic mastitis pathogen. Because our detection method was focused on MR staphylococci, the role of other mastitis pathogens as a cause of elevated SCC remains unclear. *Staphylococcus chromogenes*, which is the most frequently detected mastitis-causing NAS pathogen worldwide, was not detected in our study, in which only presumably MR-NAS were analyzed. Previous studies reported that *Staph. chromogenes* harbors low numbers of resistance genes, and MR *Staph. chromogenes* was rarely detected in samples from dairy cows (Cicconi-Hogan et al., 2014; Nobrega et al., 2018; Wuytack et al., 2020a). Missing *Staph. chromogenes* isolates from dairy cows in this study is therefore probably caused by the cefoxitin selection criterion. It remains unclear why *Staph. chromogenes* is the predominant NAS species isolated from mastitis

milk samples worldwide. Broad-spectrum β-lactam resistance is probably not the driver for natural selection of *Staph. chromogenes* as a mastitis-causing pathogen.

The detection of predominant MR-NAS species within dairy farms suggests a possible contagious transmission (Figure 1). On farm number 12, MR *Staph. sciuri* (n = 21) was the only species isolated from QMS and additionally in samples from teat liners and young stock. Similarly, predominant species were detected on farms number 4 (*Staph. sciuri*) and number 10 (*Staph. lentus*). Contagious transmission may have also occurred on farms numbers 1 and 7, where high numbers of *Staph. cohnii* isolates were detected in QMS (n = 25 and 27, respectively). The *Staph. cohnii* isolates showed a reduced susceptibility to cefoxitin but did not carry the *mecA* or *mecC* gene and were therefore not considered MR-NAS and not included in Figure 1. On most of the remaining farms (e.g., numbers 1, 3, 5, 11, 13, and 18), multiple different MR-NAS species were detected. Therefore, a contagious transmission of MR-NAS seems unlikely within these farms.

The most common MR-NAS species from calves in our study were MR *Staph. sciuri* and MR *Staph. lentus*. In a study from Belgium, MR *Staph. lentus* was not detected in swab samples from dairy calves, but it was the most common MR-NAS species in nasal swabs from veal calves (Vanderhaeghen et al., 2013). The most common MR-NAS species from environmental samples in our study were MR *Staph. sciuri* (n = 10) and MR *Staph. lentus* (n = 3). To date, no studies were performed that investigated methicillin resistance in NAS from the environment of dairy farms. In dust samples from pig barns, MR *Staph. sciuri* has been isolated before (Tulinski et al., 2012). In studies that did not include resistance testing, *Staph. sciuri* was frequently isolated from environmental samples, which is in line with our results (Piessens et al., 2011; De Visscher et al., 2014). Additional environmental NAS species on dairy farms from different studies were *Staph. fleurettii*, *Staph. equorum*, and *Staph. haemolyticus* (De Visscher et al., 2014; Jenkins et al., 2019). In our study, MR *Staph. haemolyticus* was detected in only 1 dust sample and MR *Staph. fleurettii* in teat liners from 1 farm.

In the framework of our field study, we recently reported the occurrence of MRSA in the 20 preselected study farms (Schnitt et al., 2020). In BTM, the proportion of MRSA-positive samples (63.2%; 12/19) was slightly higher than the proportion of MR-NAS-positive BTM samples (42.1%; 8/19). Except for BTM, the MR-NAS positive test rate was similar or higher in all sample types compared with the MRSA positive test rate from our study. The MRSA positive test rate in QMS from preselected cows was 2.9% (67/2,347), and the MR-NAS positive test rate was 3.3% (77/2,347; Schnitt et al., 2020). In addition, some MR-NAS species might have gone undetected in this study due to the cefoxitin-based selection procedure. This indicates that on dairy farms with an MRSA history, MR-NAS are equally or more prevalent than MRSA. This is not surprising because the classification of *Staph. aureus* and NAS is based on their pathogenic potential, and NAS consist of various staphylococcal species compared with *Staph. aureus* as a single pathogen. The effect of MRSA on the SCC in QMS (geometric mean: 345,000 cells/mL) was higher compared with MR-NAS-affected quarters (geometric mean: 183,000 cells/mL). This finding underlines the role of NAS (MR-NAS) as mastitis-causing pathogens of minor importance compared with *Staph. aureus* (MRSA).

The resistance mechanisms and related genes that mediate reduced cefoxitin susceptibility in the NAS species from Table 3 that did not carry the *mecA* or *mecC* gene remain unknown. The genetic background of cefoxitin and methicillin resistance in NAS is more diverse compared with *Staph. aureus*, and expression of the *mecA* gene is heterogeneous across NAS species (Becker et al., 2014; Humphries et al., 2020). Therefore, *mecA*- and *mecC*-negative NAS that exhibit reduced susceptibility to cefoxitin and oxacillin should be further investigated for genotypic resistance mechanisms. Previous studies reported a hyperproduction of β-lactamase (Argudín et al., 2018; Scholtzek et al., 2019) and different modifications in the penicillin-binding protein as a cause of broad-spectrum β-lactam resistance in *Staph. aureus* (Chambers, 1997).

The role of MR-NAS as a potential reservoir of resistance genes that may be transferred to methicillin-sensitive *Staph. aureus* has been discussed in numerous studies, and relatively little is known about the underlying mechanisms in vivo (Haaber et al., 2016; Miragaia, 2018; Fisher and Paterson, 2020). In the laboratory, transfer of SCC*mec* elements has been achieved by conjugation (plasmids), transduction (phages), and transformation (DNA uptake from the environment; Morikawa et al., 2012; Chlebowicz et al., 2014; Ray et al., 2016). In samples from 6 farms in our study, MR *Staph. haemolyticus* were detected that carried the same SCC*mec* type (V) as the MRSA on these farms. On one more farm, SCC*mec* type V was detected in MR *Staph. cohnii*, MRSE, and MRSA. It might be hypothesized that SCC*mec* elements have been transmitted from methicillin-resistant to susceptible staphylococcal species on these farms, leading to a higher number of resistant strains. To further investigate the similarity between the SCC*mec* elements and thus give hints for transmission events, in-depth analyses of the SCC*mec* DNA sequences should be performed. Because the majority of SCC*mec* types in MR-NAS (86.9%; 193/222)

could not be characterized in our study, whereas most MRSA strains carried SCC*mec* type V, a recent transmission of SCC*mec* elements seems unlikely (Schnitt et al., 2020). Different SCC*mec* types in NAS and *Staph. aureus* from dairy farms were previously reported (Vanderhaeghen et al., 2013). However, a possible transfer of the *mec* gene complex, independent of the SCC*mec* cassette, was also described in NAS from BTM of dairy farms (Fisher and Paterson, 2020).

CONCLUSIONS

Methicillin-resistant NAS were detected in different age groups of cattle and in the environment of 20 dairy farms that were preselected based on previous MRSA findings. The most frequently detected MR-NAS species from dairy farms was MR *Staph. sciuri*; MR *Staph. lentus* was the second most common species, especially in samples from calves. Additional MR-NAS species that were repeatedly detected in our study were *Staph. fleurettii*, *Staph. epidermidis*, and *Staph. haemolyticus*. On 15/20 farms, high numbers of *Staph. cohnii* (n = 120) ere detected in QMS that showed a reduced susceptibility to cefoxitin but did not carry the *mecA* or *mecC* genes. The QMS with high SCC were more likely to carry MR-NAS compared with all QMS included in our study, indicating a small but significant effect of MR-NAS on udder health. The MR-NAS positive test rate in samples from dairy farms was higher compared with the MRSA positive test rate from our study. This is important because resistance genes can be transferred between MR-NAS and the major mastitis pathogen *Staph. aureus*.

ACKNOWLEDGMENTS

This study was carried out in the framework of the project #1HealthPREVENT, funded by the German Federal Ministry of Education and Research (Bonn, Germany), grant no. 01KI1727C. We thank all farmers who voluntarily participated in the study. We also thank Ulrike Sorge (Tiergesundheitsdienst Bayern e.V., Poing, Germany), Thomas Peters (MBFG mbH, Wunsdorf, Germany), Karsten Donat (Thüringer Tierseuchenkasse, Jena, Germany), Sabine Reinhold (Tierärztliche Praxis am Weinberg, Jessen, Germany), Oliver Claushues (Landeskontrollverband Nordrhein-Westfalen e.V., Krefeld, Germany), Karin Eulenberger (Sächsische Tierseuchenkasse, Dresden, Germany), and Natalie Morgenstern (Landesuntersuchungsanstalt Sachsen, Dresden, Germany) for supporting the identification of study farms. The authors declare no conflicts of interest.

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3.4 Publication 4

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Genomic distinctions of LA-MRSA ST398 on dairy farms from different German federal states with a low risk of severe human infections.

https://doi.org/10.3389/fmicb.2020.575321

Frontiers in Microbiology

January 2021; 11:575321.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 08 January 2021
doi: 10.3389/fmicb.2020.575321

Genomic Distinctions of LA-MRSA ST398 on Dairy Farms From Different German Federal States With a Low Risk of Severe Human Infections

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OPEN ACCESS

Edited by:
Kristina Kadlec, Independent Researcher, Wunstorf, Germany

Reviewed by:
Phil Giffard, Menzies School of Health Research, Charles Darwin University, Australia
Renáta Karpíšková, Veterinary Research Institute (VRI), Czechia

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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *23 June 2020*
Accepted: *08 December 2020*
Published: *08 January 2021*

Citation:
Lienen T, Schnitt A, Hammerl JA, Maurischat S and Tenhagen B-A (2021) Genomic Distinctions of LA-MRSA ST398 on Dairy Farms From Different German Federal States With a Low Risk of Severe Human Infections. Front. Microbiol. 11:575321. doi: 10.3389/fmicb.2020.575321

Methicillin-resistant *Staphylococcus aureus* (MRSA) have been found on German dairy farms and may be the cause of difficult-to-treat bovine mastitis. Considering the one health approach, MRSA might be transmitted from animals to humans raising the risk for severe infections. On 17 German dairy farms with a history of MRSA detection, MRSA strains were isolated from quarter milk, bulk tank milk, and swab samples of calves, heifers, pigs, and the environment. A selection of 33 isolates was analyzed using whole-genome sequencing and antimicrobial resistance testing. All detected MRSA strains were attributed to the livestock-associated sequence type 398. Methicillin-resistance was associated with the *mecA* gene in the staphylococcal cassette chromosome (SCC)*mec* types IVa (7/33) or V (26/33). The MRSA strains across the German federal states showed large allelic differences indicating independent development and distribution. On one farm, a clonal MRSA isolate was widely spread among different animals and the milking equipment. Moreover, MRSA transmission between two dairy farms in one federal state seems to be likely. In depth studies indicated that the resistance gene prediction and phenotypic resistance are in good agreement. Twenty eight strains were determined to exhibit a non-wildtype phenotype (resistant) against up to seven antimicrobial substances with an overall resistance to β-lactams and tetracycline. Ten different phenotypic antimicrobial resistance patterns were found among the MRSA strains. The strains harbored a wide virulence gene repertoire, of which some of them are related to bovine mastitis. However, the isolates lacked typical human infection associated factors such as the immune evasion cluster genes, staphylococcal enterotoxin genes, or Panton-Valentine leukocidin genes leading to the assumption for a low risk for severe human infections and foodborne diseases.

Keywords: LA-MRSA, dairy farms, phylogenetic relationship, antimicrobial resistance, one health

INTRODUCTION

Methicillin-resistant *Staphylococcus* (*S.*) *aureus* (MRSA) were repeatedly detected on German dairy farms (Tenhagen et al., 2018; Kadlec et al., 2019) and may be a cause of bovine mastitis (Holmes and Zadoks, 2011). MRSA infections are hard to cure since these bacteria are resistant against β-lactam antibiotics, which are widely used for *S. aureus* mastitis treatment. In addition

to the animal health aspect, MRSA may be transmitted from animals to the farm personnel and sporadically cause severe infections in humans such as dermatitis, otitis, wound infection, pneumonia, endocarditis, or sepsis (Goerge et al., 2017). MRSA may carry resistance genes against several classes of antibiotics and even resistance against last resort antibiotics such as linezolid was found in isolates from various livestock (Cuny et al., 2017). Moreover, MRSA can be equipped with a wide arsenal of virulence factors such as immune evasion clusters (IECs), toxins, or leukocidins. Both, antibiotic resistance and virulence genes, are often encoded on mobile genetic elements (MGEs) giving the possibility to spread resistance or virulence between different strains. The most common MGE in MRSA with regard to antibiotic resistance is the staphylococcal cassette chromosome (SCC)*mec* element, in which the β-lactam antibiotic resistance gene *mecA* or its homolog *mecC* is located. The SCC*mec* is structurally divided into the types I–XIII (Lakhundi and Zhang, 2018). Moreover, antimicrobial resistance determinants may also be encoded on plasmids (Feßler et al., 2018). Likewise, also virulence factors are found in MGEs across the MRSA genome, e.g., in *S. aureus* pathogenicity islands (SaPIs) or phages. Livestock-associated MRSA (LA-MRSA) often lack the potential for causing severe human infections due to a lack of IEC genes or genes encoding the toxic shock syndrome toxin (TSST) or Panton-Valentine leucocidin (PVL; Cuny et al., 2015a). However, frequent monitoring of LA-MRSA strains from different livestock farms and the respective environment are necessary, since the genetic repertoire of MRSA strains might change spontaneously due to horizontal gene transfer leading to more harming strains with regard to animal and human health (Kraushaar et al., 2017).

The aim of the study was to compare the genotypes, antimicrobial resistance profiles, and virulence factors of MRSA strains from 17 dairy farms in eight German federal states. Whole-genome sequencing (WGS) of selected strains was conducted and the sequence data were analyzed regarding antimicrobial resistance genes and virulence factors to draw conclusions for a potential public health risk. Furthermore, the phylogenetic relationship between MRSA strains from various regions as well as within one farm was analyzed by core genome multi-locus sequence typing (cgMLST).

MATERIALS AND METHODS

Sampling and MRSA Strain Selection

For this study, 17 dairy farms across eight German federal states were selected due to a previous positive MRSA detection. Samples from bovine mammary quarters (quarter milk samples, QMS), bulk tank milk (BTM), calves (nasal swabs), heifers (nasal swabs and udder cleft swabs), pigs (nasal swabs) on dairy farms as well as the milking equipment and environment, which were retrieved in a sampling campaign from September 2018 to December 2019, were examined for MRSA. Milk (1 ml) and swab samples were examined using a double selective enrichment method by incubation in Mueller Hinton broth (Thermo Fisher Scientific Oxoid Ltd., United Kingdom) supplemented with 6% of NaCl, tryptic soy broth (Merck, Germany) supplemented with 3.5 mg/l cefoxitin (Sigma-Aldrich, United States) and 50 mg/l aztreonam (Sigma-Aldrich, United States) and subsequent incubation on mannitol salt (Thermo Fisher Scientific Oxoid Ltd., United Kingdom) agar plates containing 4 mg/l cefoxitin (Sigma-Aldrich, United States). Each incubation step lasted for 24 ± 2 h at 37°C. With regard to a potential food intoxication or transmission to humans by the consumption of MRSA contaminated milk, in particular strains from QMS and BTM were chosen for sequencing if available. In total, 33 out of 184 MRSA isolates were selected as most interesting for comparison by WGS according to previous PCR results with regard to SCC*mec* type and *spa* type (Schnitt et al., 2020). In **Table 1**, all sequenced MRSA strains are listed. The strains originated from QMS, BTM, nasal swabs of calves, heifers, and a pig as well as a swab from a teatcup and a teat cleaning water sample. The data were anonymized due to the general data protection regulation. The code is a combination of the German federal state, the farm in the respective federal state and the sample number of the respective farm, e.g., AA1 means German federal state A, farm A from this federal state and sample number 1 from this farm. For studying the transmission of MRSA strains across one farm, isolated strains from various sample types were included from farm AA.

DNA Extraction and WGS

Methicillin-resistant *Staphylococcus aureus* isolates were cultured on sheep blood agar (Oxoid GmbH, 46483, Wesel, Germany) and DNA of one inoculation loop filled with MRSA colonies was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol modified by adding 10 µl lysostaphin to the lysis buffer. The DNA library was prepared using an Illumina Nextera DNA Flex kit (Illumina Inc., United States) and the 150 bp paired-end sequencing run was performed on an Illumina NextSeq 500 instrument.

Bioinformatic Analyses

Assembly and Quality Control

Raw Illumina reads were trimmed and *de novo* assembled with the in-house developed Aquamis pipeline[1] which implements fastp (Chen et al., 2018) for trimming and shovill (based on SPAdes)[2] for assembly. Furthermore, it performs mash v 2.1 for reference search (Ondov et al., 2016) as well as quast v 5.0.2 for assembly quality control (Mikheenko et al., 2018). The minimal coverage depth was >80. Quality of assemblies was checked by single-copy and duplicated orthologs analyses. The fraction majority species was >0.97. The total genome length was >2.7 Mbp.

Phylogenetic Analyses

The MLST sequence type was inferred using mlst[3] with the pubmlst database (Jolley and Maiden, 2010). Moreover, SCC*mec*- and *spa*-types were predicted with respect to the software tools SCCmecFinder 1.2 and spaTyper 1.0 of the Centre for Genomic

[1]https://gitlab.com/bfr_bioinformatics/AQUAMIS/

[2]https://github.com/tseemann/shovill

[3]https://github.com/tseemann/mlst

TABLE 1 | Overview of sequenced MRSA strains, source, SCC*mec*-, *spa*-type, ST, prediction of antimicrobial resistance genes, and phenotypic resistance.

Nr	Code	Source	SCC*mec*-type	*spa*-type	ST[5]	Predicted antimicrobial resistance genes	Phenotypic resistance[6]
1	AA1	QMS[1]	IVa	t011	398	*aac(6')Ie-aph(2")Ia;blaZ;dfrK;mecA;str;tet*(M)	FOX, GEN, KAN, PEN, STR, TET, TMP
2	AA2	QMS	IVa	t011	398	*aac(6')Ie-aph(2")Ia;blaZ;dfrK;mecA;str;tet*(M)	FOX, GEN, KAN, PEN, STR, TET, TMP
3	AA3	QMS	IVa	t011	398	*aac(6')Ie-aph(2")Ia;blaZ;dfrK;mecA;str;tet*(M)	FOX, GEN, KAN, PEN, STR, TET, TMP
4	AA4	Pig	V	t1451	398	*blaZ;erm*(A)*;mecA;tet*(M)*;spc;vga*(E)	FOX, ERY, PEN, TET, TIA
5	AA5	Calf	IVa	t011	398	*aac(6')Ie-aph(2")Ia;blaZ;dfrK;mecA;tet(M)*	FOX, GEN, KAN, PEN, STR, TET, TMP
6	AA6	Heifer	V	t1451	398	*blaZ;erm*(A)*;mecA;tet*(M)*;spc;vga*(E)	FOX, ERY, PEN, TET, TIA
7	AA7	TC[2]	IVa	t011	398	*aac(6')Ie-aph(2")Ia;blaZ;dfrK;mecA;str;tet*(M)	FOX, GEN, KAN, PEN, STR, TET, TMP
8	AA8	BTM[3]	IVa	t011	398	*aac(6')Ie-aph(2")Ia;blaZ;dfrK;mecA;str;tet*(M)	FOX, GEN, KAN, PEN, STR, TET, TMP
9	AA9	TCW[4]	IVa	t011	398	*aac(6')Ie-aph(2")Ia;blaZ;dfrK;mecA;str;tet*(M)	FOX, GEN, KAN, PEN, STR, TET, TMP
10	BA1	QMS	V	t011	398	*blaZ;mecA;str;tet*(K)*;tet*(M)*;vga*(A)	FOX, PEN, STR, TET, TIA
11	BB1	QMS	V	t034	398	*dfrG;erm*(A)*;mecA;tet*(K)*;tet*(M)*;spc;vga*(E)	FOX, CLI, ERY, PEN, TET, TIA, TMP
12	BC1	QMS	V	t011	398	*blaZ;mecA;tet*(K)*;tet*(M)	FOX, PEN, TET
13	BC2	BTM	V	t011	398	*blaZ;mecA;tet*(K)*;tet*(M)	FOX, PEN, TET
14	CA1	QMS	V	t034	398	*dfrG;lnu*(B)*;lsa*(E)*;mecA;tet*(K)*;tet*(M)*; spc*	FOX, CLI, PEN, Q–D, TET, TIA, TMP
15	CB1	QMS	V	t011	398	*blaZ;mecA;tet*(K)*;tet*(M)	FOX, PEN, TET
16	DA1	Calf	V	t011	398	*blaZ;mecA;tet*(K)*;tet*(M)	FOX, PEN, TET
17	DB1	Calf	V	t571	398	*dfrG;erm*(A)*;mecA;tet*(K)*;tet*(M)*;spc;vga*(E)	FOX, CLI, ERY, PEN, TET, TIA, TMP
18	EA1	BTM	V	t011	398	*blaZ;mecA;tet*(K)*;tet*(M)*;vga*(A)	FOX, PEN, TET, TIA
19	EB1	QMS	V	t034	398	*blaZ;dfrG;mecA;tet*(K)*;tet*(M)*;vga*(A)	FOX, PEN, TET, TIA, TMP
20	EB2	QMS	V	t034	398	*blaZ;dfrG;mecA;tet*(K)*;tet*(M)*;vga*(A)	FOX, PEN, TET, TIA, TMP
21	EC1	QMS	V	t034	398	*blaZ;dfrG;mecA;tet(M);vga*(A)	FOX, CLI, PEN, TET, TIA, TMP
22	EC2	QMS	V	t011	398	*blaZ;dfrG;mecA;tet*(K)*;tet*(M)*;vga*(A)	FOX, PEN, TET, [TIA], TMP
23	ED1	Heifer	V	t034	398	*blaZ;dfrG;mecA;tet*(K)*;tet*(M)*;vga*(A)	FOX, PEN, TET, TIA, TMP
24	ED2	BTM	V	t1928	398	*blaZ;dfrG;mecA;tet*(K)*;tet*(M)*;vga*(A)	FOX, PEN, TET, TIA, TMP
25	EE1	QMS	V	t011	398	*blaZ;dfrG;mecA;tet*(K)*;tet*(M)*;vga*(A)	FOX, PEN, TET, [TIA], TMP
26	EE2	QMS	V	t011	398	*blaZ;dfrG;mecA;tet*(K)*;tet*(M)*;vga*(A)	FOX, PEN, TET, TIA, TMP
27	EE3	QMS	V	t011	398	*blaZ;dfrG;mecA;tet*(K)*;tet*(M)*;vga*(A)	FOX, PEN, TET, TIA, TMP
28	EE4	QMS	V	t1928	398	*blaZ;dfrG;mecA;tet*(K)*;tet*(M)*;vga*(A)	FOX, PEN, TET, TIA, TMP
29	EE5	QMS	V	t1928	398	*blaZ;dfrG;mecA;tet*(K)*;tet*(M)*;vga*(A)	FOX, PEN, TET, [TIA], TMP
30	FA1	Calf	V	t034	398	*blaZ;dfrG;fexA;mecA;tet*(K)*;tet*(M)	FOX, CHL, PEN, TET, TMP
31	FB1	QMS	V	t2011	398	*blaZ;mecA;tet*(K)*;tet*(M)	FOX, PEN, TET
32	GA1	BTM	V	t034	398	*dfrG;erm*(A)*;mecA;tet*(K)*;spc;vga*(E)	FOX, CLI, ERY, PEN, TET, TIA, TMP
33	HA1	Calf	V	t034	398	*blaZ;dfrG;mecA;tet*(K)*;tet*(M)	FOX, PEN, TET, TMP

Underlined resistances were not predicted. Resistances in brackets were predicted but not detected phenotypically. [1]QMS, Quarter milk sample. [2]TC, Teat cup. [3]BTM, Bulk tank milk. [4]TCW, Teat cleaning water. [5]ST, Sequence type. [6]FOX, Cefoxitin; GEN, Gentamycin; KAN, Kanamycin; PEN, Penicillin; STR, Streptomycin; TET, Tetracycline; TMP, Trimethoprim; CLI, Clindamycin; ERY, Erythromycin; TIA, Tiamulin; Q–D, Quinupristin–Dalfopristin; CHL, Chloramphenicol.

Epidemiology.[4] In addition, the phylogenetic relationship of all sequenced MRSA strains was analyzed using cgMLST in Ridom SeqSphere + version 7.0.4. The default settings were kept so that clusters were defined at less than 24 allelic differences.

AMR Genes

Bacterial characterization was conducted with the in-house developed Bakcharak pipeline,[5] which implements ABRicate[6] for screening of antimicrobial resistance genes using the NCBI amrfinder database (Feldgarden et al., 2019).

Virulence Factor Genes

Virulence factor genes were predicted using the VFDB (Chen et al., 2005). Following the staphylococcal virulence factor classification of Naushad et al. (2019), the detected virulence factor genes were attributed to the functional categories adhesion, exoenzymes, hemolysis, immune evasion, iron uptake, and metabolism or secretion. A detailed sequence search for SaPIs and phages in the obtained sequences was performed using the NCBI blastn suite. Therefore, a collection of SaPIs and phages (phiNM3, phi80, phiPVL, phiETA, Saeq1, SaPI1-3, SaPIbov1-2, SaPIbov4-5, SaPIeq1, SaPIivm10, SaPIishikawa11, SaPIivm60, SaPIino10, SaPIhirosaki4, SaPIj11, SaPIhhms2, SaPINN54, SaPIPM1, SaPI68111, and SaPIj50) was chosen according to the publications of Walther et al. (2018) and Alibayov et al. (2014).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) was performed by broth microdilution according to the CLSI standard (ISO 20776-1:2006 or CLSI M31-A3) using a standardized antibiotic panel (EUVENC scheme) that is used in all member states of the

[4]https://cge.cbs.dtu.dk/services/

[5]https://gitlab.com/bfr_bioinformatics/bakcharak

[6]https://github.com/tseemann/abricate

European Union for resistance monitoring on staphylococci from livestock and food. For evaluation of minimal inhibitory concentrations (MIC) of the individual isolates the clinical breakpoints values of the CLSI were used. For quality control of resistance testing the *S. aureus* isolates ATCC 29213 and ATCC 25923 were used.

RESULTS

MLST-, SCC*mec*-, and *spa*-Typing of MRSA Strains

Analyses of the sequence data showed that all strains belonged to the sequence type (ST) 398 (**Table 1**). MRSA strains with SCC*mec* type V dominated on the dairy farms. Solely on one farm AA, MRSA strains with SCC*mec* type IVa were detected in BTM, QMS, a nasal swab of a calf, a teatcup, and the teat cleaning water, whereas the MRSA strains AA4 (pig) and AA6 (heifer) from the animals placed in the pig barn carried SCC*mec* type V. Regarding the *spa*-types, t011, and t034 were mostly found. Beside, also *spa*-types t1451, t571, t1928, and t2011 were detected. On four farms (AA, EC, ED, and EE) various *spa*-types were found in different sample types. On farm AA and in accordance with the varying SCC*mec* types, *spa*-type t011 was found in BTM, QMS, the nasal swab of a calf, the teatcup and the teat cleaning water, whereas the MRSA strains from the nasal swabs of a pig and a heifer located in the pig barn carried *spa*-type t1451. On farm EC, two different *spa*-types (t011 and t034) were found in QMS. The *spa*-types t034 (nasal swab of heifer) and t1928 (BTM) were detected on farm ED. Moreover, on farm EE, different *spa*-types (t011 and t1928) were found in QMS.

Antimicrobial Resistance Profiles

A broad range of antimicrobial resistance genes was detected in the sequences of the different MRSA strains. Resistance to the antibiotic classes aminoglycoside, β-lactam, trimethoprim, tetracycline, macrolide, streptogramin, lincosamide, and phenicol were predicted showing differences between the dairy farms and sample types on farm AA (**Table 1**). All MRSA strains (33/33) carried the *mecA* gene, whereas the β-lactamase encoding *blaZ* gene was missing in four strains. Resistance to the other antibiotic classes was encoded by the following genes; aminoglycoside [*aac(6')Ie-aph(2")Ia, str*], aminocyclitol (*spc*), macrolide-lincosamide-streptogramin B [*erm*(A)], trimethoprim (*dfrG, dfrK*), tetracycline [*tet*(K), *tet*(M)], pleuromutilin–lincosamide–streptogramin A [*lsa*(E), *vga*(A), *vga*(E)], lincosamides [*lnu*(B)], and phenicol (*fexA*). All MRSA strains (33/33) harbored tetracycline resistance genes. Resistance to aminoglycosides (14/33), trimethoprim (24/33), and pleuromutilin–lincosamide–streptogramin A (19/33) was also commonly predicted. In contrast, resistance to macrolides–lincosamide–streptogramin B (5/33) and phenicol (1/33) was less frequently predicted.

The phenotypic resistance according to the MIC values was in good agreement with the predicted antimicrobial resistance genes (**Table 1**). The genotypic differences between the strains throughout the farms were also shown in the phenotypic resistance pattern. All MRSA strains were resistant to cefoxitin, penicillin, and tetracycline. Resistance to trimethoprim and tiamulin was also widespread. Only a few strains showed resistance to gentamicin (7/33), kanamycin (7/33), streptomycin (8/33), clindamycin (5/33), or erythromycin (5/33). Only the strain CA1 was phenotypically resistant to quinupristin–dalfopristin and strain FA1 was phenotypically resistant to chloramphenicol. In sum, ten different phenotypic resistance patterns occurred within the various MRSA strains.

Virulence Factors

The MRSA strains showed a diverse repertoire of virulence factor genes. Most of the analyzed genes (45/63) were present in every MRSA strain (**Table 2**). The *cna* (6/33), *sdrE* (32/33), and von Willebrand factor binding protein (32/33) genes were not found in all strains. Typical human MRSA IEC genes such as *scn*, *sak,* and *chp* or exfoliative toxin genes (*eta*/*etb*) were not detected in any of the sequenced isolates. Likewise, genes encoding toxins such as staphylococcal enterotoxins (SE; *sea, seb, sec, sed, see, seh, selk, sell, selq*) or TSST (*tsst*) were not found. Regarding PVL, the leukocidin subunit *lukF-PV* gene was only detected in seven MRSA strains, whereas the *lukS-PV* gene, which encodes the other PVL subunit, was not found. The pathogenicity island SaPIbov5 was detected in eight MRSA strains. Seven of these were isolates from the same farm AA. SaPIbov4 was found in eleven strains; with a sequence coverage regarding the reference of 89–90%. Other SaPIs or phages were not detected in the assembled sequences.

Phylogenetic Relationship

The sequences were analyzed by cgMLST regarding the phylogenetic relationship of the MRSA strains from the dairy farms across the eight German federal states. Four different clusters were retrieved in the minimum spanning tree (MST) analysis (**Figure 1**). The MST analysis showed large allelic differences between the MRSA strains from different German federal states and also between farms from the same region. Moreover, MRSA strains from the same farm (EB, EC, and EE) sometimes differed significantly in the core genome. The MRSA strains EB1, ED1/2, and EE1/2/4/5 from three farms from German federal state E (cluster 2) clustered with 13–23 alleles differences closer together in comparison to the farms from other federal states. On farm AA, MRSA strains of the sample types QMS (AA1–3), BTM (AA8), the nasal swab of a calf (AA5) as well as the samples of the teatcup (AA7) and teat cleaning water (AA9) clustered closely together with a maximum difference of one core genome allele. Contrary to this, the strains AA4 (nasal swab of pig) and AA6 (nasal swab of heifer), with a pig barn origin, formed an own cluster, which is in accordance with the different SCC*mec*- and *spa*-types.

DISCUSSION

Methicillin-resistant *Staphylococcus aureus* may be widespread on German dairy farms potentially causing infections such as bovine mastitis (Schnitt and Tenhagen, 2019). In addition to

TABLE 2 | Number of MRSA strains harboring various predicted virulence associated genes and corresponding functional categories.

Function	Predicted virulence factor genes	No. of strains
Adhesion	*cap, clfA/B, ebp, fnbA, map, sdrC/D*	33 (100%)
	can	6 (18%)
	sdrE	32 (97%)
Biofilm formation	*icaA/B/C/D/R*	33 (100%)
Exoenzymes	*aur, geh, lip, hysA, sspA/B/C*	33 (100%)
	von Willebrand factor binding protein	32 (97%)
Hemolysis	*hla, hlb, hld, hlgA/B/C*	33 (100%)
Immune evasion	*coa, spa, sbi*	33 (100%)
Iron uptake and metabolism	*isdA/B/C/D/E/F/G, srtB*	33 (100%)
Secretion	*esaA/B/C, essA/B/C, esxA/B*	33 (100%)
Toxin	*lukF-PV*	7 (21%)
	lukS-PV	0
	tsst	0
	sea, seb, sec, sed, see, seh, selk, sell, selq	0
IEC[1]	*chp, scn, sak*	0
MGE[2]	SaPIbov4	11 (33%)
	SaPIbov5	8 (24%)

[1] *IEC, Immune evasion cluster.* [2] *MGE, Mobile genetic element.*

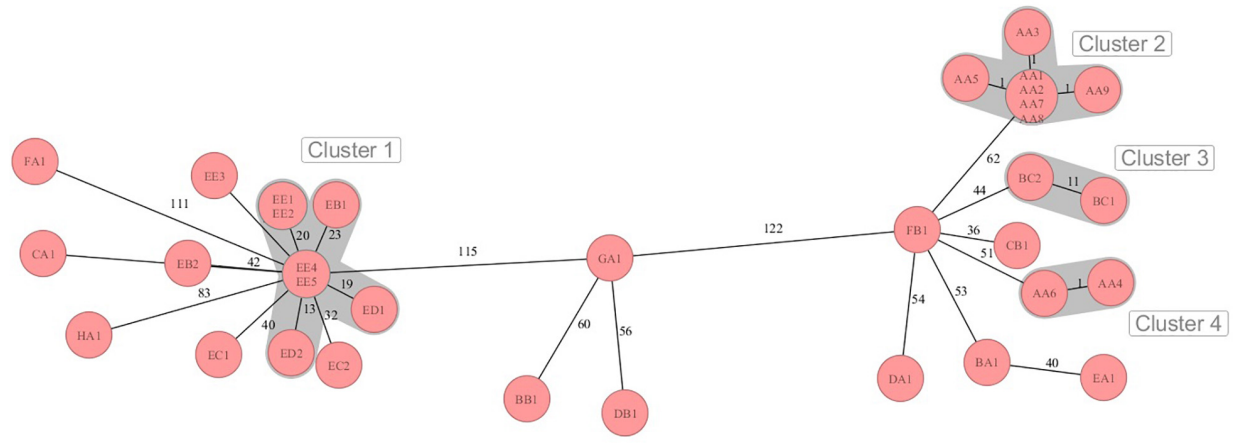

FIGURE 1 | Minimum spanning tree visualization of cgMLST analysis of the MRSA strains on dairy farms from eight German federal states A-H with four clusters of close phylogenetic relationship. Numbers represent the allelic differences between the MRSA strains.

animal health and with regard to a transmission from animals to humans, monitoring of MRSA abundance and genotyping is also important in the public health context.

Genomic Distinctions Between MRSA Isolates Across Germany

In our study, 33 MRSA strains from 17 dairy farms across eight German federal states were phenotypically and genotypically analyzed. All strains were characterized as ST398 LA-MRSA, a sequence type, which is often found on dairy farms (Feßler et al., 2012; Kadlec et al., 2019) and which is the most widely disseminated LA-MRSA sequence type (Cuny et al., 2015b). SCC*mec*-types IV and V as well as the *spa*-type t011 dominated in LA-MRSA on Dutch dairy farms as reported by Feßler et al. (2012). This is in accordance with the results of our study, however, in contrast, SCC*mec*-type IVa was only found in 1/17 farms and the *spa*-type t034 co-dominated to the *spa*-type t011. Tenhagen et al. (2018) also detected the *spa*-types t011 and t034 as most frequent in MRSA of BTM from German dairy farms. Moreover, a dominance of MRSA carrying SCC*mec* type V on German dairy farms was shown in the study of Kadlec et al. (2019).

The phylogenetic analysis of the MRSA strains done by cgMLST showed mainly distinct allelic differences and only a few clusters of close relationship. Although the strains were similar according to SCC*mec*- or *spa*-typing, the core genome differed in some cases by more than 120 alleles. This illustrates the high genomic plasticity and strong evolution in terms of genetic recombination in MRSA. Although exhibiting differences

of 56–60 alleles, the strains GA1, BB1, and DB1 clustered together in the MST. The phylogenetic relation of these strains was also indicated by the similar antimicrobial resistance genes profile and the identical phenotypic resistance pattern. Only some MRSA strains from federal state E showed lower genetic divergence, in particular the strains ED2 and EE4/5. These strains also shared the same *spa*-type t1928. It can be speculated that a transmission of these MRSA strains between the farms took place. Reasons for this might be humans, e.g., farm personnel or veterinarians, or animal trade transmitting MRSA from one farm to another. In line with that, the introduction and transmission of MRSA on pig farms in Norway by farm workers or livestock trade was illustrated in the studies of Grontvedt et al. (2016) and Elstrøm et al. (2019). The potential role of "humans" as MRSA vectors across dairy farms was also shown in a recently published review of Schnitt and Tenhagen (2019).

Since the MRSA genotype might also vary within farms, strains of different sample types from selected farms were investigated. MRSA strains with a maximum of one allele difference in cgMLST were found in BTM, QMS of different cows, a nasal swab of a calf, the teatcup of the milking equipment and water for teat cleaning prior to milking on farm AA. Moreover, the strains harbored the same antimicrobial resistance genes and showed the same phenotypic resistance pattern. Therefore, the spread of one clonal LA-MRSA strain between cows, calves, and the milking equipment seems likely. In contrast, the MRSA isolates of the pig and heifer placed in the pig barn showed a MRSA strain, which differed genotypically from the strain in the dairy barn. A spillover of MRSA from the pig production to veal calves or dairy cows was suggested by several studies (Locatelli et al., 2017; Hansen et al., 2019). However, in our study, the spillover from pigs to other animals was only found in close proximity in the pig barn. Various MRSA genotypes also co-existed on farms EB, EC, and EE. Accordingly, Feßler et al. (2012) showed a co-dominance of several MRSA strains on one dairy farm. A reason for this might be the purchase of new animals and thus MRSA genotypes in the farm or transmission of additional strains by humans or other vectors. On the other side, genetic diversification might have appeared on the farm leading to altered MRSA genotypes.

Widespread Multi-Resistant MRSA

The MRSA strains in our study carried a broad repertoire of antimicrobial resistance genes and 28 strains were multi-resistant to at least three classes of antibiotics. This is contrary to the recently published study of Kadlec et al. (2019), in which less than half of the MRSA strains from German dairy farms were multi-resistant with the constraint that only ten MRSA isolates were investigated by the authors. The prediction of the antimicrobial resistance genes in our study was mostly in agreement with the phenotypic antibiotic class resistance pattern. Resistance to β-lactam antibiotics such as cefoxitin or penicillin was mediated by the *mecA* gene in all strains, whereas the variant *mecC* gene was not found. In contrast, Schlotter et al. (2014) found MRSA harboring the *mecC* gene in 16 of 56 milk samples in a German dairy herd, probably due to a spread of this strain within the farm. However, in accordance with our study, MRSA strains in dairy cattle from Germany and Greece only harbored the *mecA* gene (Kadlec et al., 2019; Papadopoulos et al., 2019). The resistance to several classes of antibiotics was mediated by two to three different genes in our study. Feßler et al. (2018) summarized in their review that small plasmids play a pivotal role in the dissemination of certain antimicrobial resistance genes. Although not analyzed in detail, the transmission of resistance genes such as *vga*(A) or *dfrK* through plasmids likely also played a role in the strains of our study. In general, the antimicrobial resistance patterns differed between the farms indicating an independent development of the strains across Germany. Resistance to macrolides was rare in the MRSA strains. Accordingly, macrolide resistance in *S. aureus* retrieved from bovine mastitis was reported to be low (Pyorala et al., 2014; El Garch et al., 2020). In contrast, in a study of Tenhagen et al. (2018) macrolide resistance was detected in 17/41 MRSA isolates of BTM from German dairy farms. In our study, resistance to chloramphenicol was only found in one MRSA strain harboring the *fexA* gene. Accordingly, the *fexA* gene was only rarely found in a study analyzing ST398 MRSA isolates from bovine mastitis (Feßler et al., 2010). Resistance to tetracyclines was found in every strain from all farms in our study mediated by the *tet*(K) or *tet*(M) genes. In agreement with this, in the studies of Feßler et al. (2012) and Kadlec et al. (2019) all MRSA strains from dairy farms were resistant to tetracycline. Moreover, tetracycline resistance was detected in 99.4% of the MRSA isolates received from the cattle food chain (Tenhagen et al., 2014) and in 95.1% of the MRSA in BTM from German dairy farms (Tenhagen et al., 2018). Tetracyclines have been extensively used on animal farms, thus promoting the survival of tetracycline resistant strains (Granados-Chinchilla and Rodriguez, 2017). Furthermore, resistance to trimethoprim and tiamulin, a pleuromutilin, was detected in more than half of the MRSA strains in our study. Staphylococci are non-target bacteria with respect to pleuromutilins, however, their use in especially pig farming selects for multi-resistant MRSA (van Duijkeren et al., 2014). In this study, pleuromutilin resistance was transmitted by the *vga*(A) or *vga*(E) genes. In particular in the German federal state E, the *vga*(A) gene was present in MRSA strains from several farms. The *vga*(A) gene was shown to be transferred by plasmids (Feßler et al., 2018), whereas the *vga*(E) gene is located on a transferable transposon (Schwendener and Perreten, 2011). Hauschild et al. (2012) originally detected the *vga*(E) gene in dairy cattle. Although the *vga*(A) gene was reported to be most widespread among the *vga* genes (Feßler et al., 2018), in our study, also the *vga*(E) gene was equally distributed across the MRSA strains from the dairy farms. The resistance to trimethoprim was mediated by the *dfrG* or *dfrK* genes. The *dfrK* gene was only detected on farm AA in the MRSA strains with SCC*mec* type IVa. This is contrary to the finding of a 85.7% dissemination of the *dfrK* gene in MRSA isolates of bovine mastitis (Feßler et al., 2010). Moreover, the physical linkage of the *dfrK* and *tet*(L) genes, as described in Kadlec et al. (2012), was not found in our study. Aminoglycosides are widely used in veterinary medicine (EMA, 2018). Only the SCC*mec* IVa MRSA strains of farm AA showed phenotypical aminoglycoside resistance to streptomycin, gentamicin, and kanamycin. This can be explained by the different repertoire of antimicrobial resistance

genes to aminoglycosides in the respective strains encoded by the *aac(6')Ie-aph(2")Ia,* and *str* genes. Moreover, the *spc* gene was detected in five strains, which mediates resistance to spectinomycin, an aminocyclitol, only (Zarate et al., 2018). The MRSA strain CA1 was phenotypically resistant to the streptogramin A and B quinupristin–dalfopristin. This is in agreement with the detection of the *lsa*(E) gene, since an eight-fold increased quinupristin–dalfopristin MIC was also previously detected for *S. aureus* harboring the *lsa*(E) gene (Wendlandt et al., 2013). Moreover, also resistance to clindamycin as detected for strain CA1 is associated with the *lsa*(E) gene (Wendlandt et al., 2013). Furthermore, *erm* genes confer inducible or constitutive resistance to macrolides, lincosamides, and streptogramin B (Leclercq, 2002). Therefore, in our study, the strains BB1 and GA1, which harbored the resistance gene *erm*(A), on the one hand showed phenotypical resistance to the macrolide erythromycin, but on the other side these strains were also resistant to the lincosamide clindamycin.

Large Repertoire of Virulence Factor Genes

The LA-MRSA strains from the dairy farms in our study harbored multiple virulence associated genes, most of them present in all strains. Piccinini et al. (2010) postulated that a specific virulence gene combination is related to the development of subclinical mastitis and the prevalence of *S. aureus* in dairy herds. Moreover, Magro et al. (2017) related some of the virulence factors to more contagious *S. aureus* strains with regard to mastitis. In accordance with the prediction of the *hlb* gene, β-hemolysis on sheep blood agar was found for every detected MRSA strain in our study (data not shown). The presence of hemolysins as a factor for bovine mastitis in Russian dairy herds was described in the study of Fursova et al. (2020). In our study, the clumping factor encoding genes *clfA* and *clfB* were detected in all strains. This is in contrast to the study of virulence factor genes in dairy cattle from Brazil conducted by Klein et al. (2012), in which the prevalence of the *clfB* gene was higher (91.8%) than the *clfA* gene prevalence (50.6%). In particular, the ClfB protein is associated with *S. aureus* nasal colonization and skin infections in humans (Wertheim et al., 2008; Lacey et al., 2019). With regard to the animal health aspect, it was related to *S. aureus* prevalence in bovine mastitis (Magro et al., 2017). The *cna* gene, which encodes a collagen adhesion protein, was found in six MRSA strains in our study. Accordingly, Klein et al. (2012) detected a *cna* gene prevalence of 22.4% in 85 MRSA isolates of dairy cattle from Brazil. *Cna* might play a pivotal role in binding collagen in wounded, injured, or inflamed tissue, e.g., in mastitis (Madani et al., 2017). In addition, in our study, the fibronectin-binding protein encoding *fnbA* gene was present in all MRSA strains. This protein was shown to be connected to mastitis in a mouse model (Brouillette et al., 2003) and it seems to be related to more contagious *S. aureus* strains in bovine mastitis (Magro et al., 2017). The *sdrC*, *sdrD*, and *sdrE* genes, which encode the serine-aspartate repeat proteins, were found in nearly all strains in our study. In particular, the presence of the *sdrD* gene was associated with bone infections (Trad et al., 2004) and more contagious *S. aureus* strains during mastitis (Magro et al., 2017). Moreover, biofilm formation plays a crucial role in virulence of *S. aureus* (Costerton et al., 1999). The genes *icaA*, *icaB*, *icaC*, *icaD,* and *icaR*, which are related to biofilm formation in several staphylococcal species, were present in all MRSA isolates in our study. Since biofilm formation is of high clinical impact (Martín-López et al., 2002), this might have also been an important issue regarding animal health in the MRSA isolates in our study.

Low Risk for Public Health

Virulence factor genes encoding the TSST, PVL, or elements of the IEC, which are associated with severe human infections, were not detected in our MRSA isolates. This is in agreement with the absence of most SaPIs and phages in the genomes, since in LA-MRSA these pathogenicity factors are often encoded in SaPIs (Ballhausen et al., 2017). SaPIbov5 and SaPIbov4, with a sequence coverage of 89–90%, were detected in 24 and 33% of our strains. Most likely, the von Willebrand factor-binding protein, a clotting factor encoded by the von Willebrand factor binding protein gene, was located on the SaPIs of the respective strains as shown by Viana et al. (2010). In particular, the SCC*mec* IVa MRSA strains from farm AA were equipped with both SaPIs. Cuny et al. (2016) associated some SCC*mec* IV and *spa*-type t011 LA-MRSA strains to human infections and Walther et al. (2018) found that 72% of ST398 and *spa*-type t011 MRSA strains in horse clinics harbored the human IEC encoded in a phiSa3 phage. Anyhow, the lack of TSST, PVL, and IEC genes in the strains from the farms investigated in our study indicates a low risk for severe human infections.

Furthermore, it has to be considered that food intoxication might appear by the consumption of raw milk or raw milk products, if SE producing MRSA strains are present in high numbers (Sergelidis and Angelidis, 2017). Yang et al. (2020) found high frequencies of SE genes in MRSA isolates of bovine mastitis cases from China. SE genes were also detected in MRSA strains of raw milk from Italy (Riva et al., 2015) and Egyptian dairy herds (El-Ashker et al., 2020). In contrast, Kadlec et al. (2019) and Kreausukon et al. (2012) did not detect any SE genes in LA-MRSA strains from German dairy farms. Accordingly, all strains investigated in our study were lacking genes for SEs, thus also lowering the possibility of a food poisoning. Moreover, the mastitis-related *S. aureus* genotype GTB, which is associated with the presence of the *sea*, *sed*, and *sej* genes (Graber et al., 2009), was not found in our study.

Our study is limited by the number of strains that were investigated with respect to the phylogenetic dynamics of MRSA strains across German dairy farms. Therefore, as a future perspective, sampling on dairy farms across Germany should be expanded and as a consequence, longitudinal core genome analysis of larger numbers of MRSA strains should be performed to better resolve the transmission pathways and evolutionary mechanisms of MRSA.

CONCLUSION

The results of our study show that MRSA on German dairy farms harbor a broad repertoire of antimicrobial resistance

and virulence factor genes. Some of the virulence genes are associated to mastitis, but none of them are connected to human infections. Phylogenetic analyses indicate more than 24 allelic differences of the strains across Germany with some regional spots of minor allelic diversity. Transmission of MRSA between farms may occur and MRSA strains may also be expansively transmitted within the farm environment. The prediction of antimicrobial resistance through bioinformatics tools was in agreement with the phenotypic resistance profiles. MRSA monitoring on animal farms is of high significance, since the genetic repertoire might spontaneously change due to horizontal gene transfer of MGEs and transmission pathways need to be resolved for containment of MRSA on animal farms.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, BioProject PRJNA634452.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because sampling of milk and nasal swabs from calves and heifers was carried out in accordance with German legislation. No ethical approval from the Institutional Ethics Committee or the National Animal Experimentation Council was required. Samples were collected by a trained veterinarian with consent from the owners of the animals. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

TL, AS, and B-AT: study design. TL, AS, and JAH: laboratory work. TL and AS: data analysis. All authors: manuscript preparation and review.

FUNDING

Our study was carried out in the framework of the project #1HealthPREVENT, funded by the German Federal Ministry of Education and Research, Grant No. 01KI1727C.

ACKNOWLEDGMENTS

We thank all farmers, who voluntarily participated in the study. Many thanks to Thomas Peters, Ulrike Sorge, Karsten Donat, Sabine Reinhold, Oliver Claushues, Karin Eulenberger and Natalie Morgenstern for supporting the identification of study farms. Special thanks to Daniel Leeser-Boek, Pascal Witt and Ylanna Kelner-Burgos for NGS preparation and Heidi Wichmann-Schauer for help with study conception. We also thank Carlus Deneke and Simon Tausch for bioinformatical analysis.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4 General discussion

Methicillin resistant staphylococci (MRS) may cause mastitis in dairy cows and pose a risk for zoonotic infections in humans. There is a lack of knowledge regarding the occurrence of MRS in other habitats on dairy farm beside the cows' milk and on possible routes of transmission. The objectives of this thesis were to 1) investigate the occurrence and transmission of MRS on dairy farms, 2) to evaluate the impact of MRS on udder health of dairy cows and 3) to discuss MRS monitoring, prevention, and control strategies.

4.1 The occurrence of methicillin resistant *Staphylococcus aureus*

A comprehensive literature review on methicillin resistant *Staphylococcus* (*S.*) *aureus* (MRSA) in dairy herds was performed (publication 1). MRSA prevalence and epidemiology as well as potential risk factors for the occurrence of MRSA in dairy herds were analyzed. MRSA were detected in quarter milk samples (QMS) and bulk tank milk (BTM) from dairy cows worldwide. In North America, MRSA prevalence seems to be rather low in dairy herds (0-1.3%) (Haran et al. 2012; Cicconi-Hogan et al. 2014). Studies from Korea and Germany provide evidence that the MRSA prevalence might be increasing in dairy herds from these countries (Song et al. 2016; Tenhagen et al. 2018). In German monitoring studies, the MRSA prevalence in BTM was 4.7% in 2010 and 9.7% in 2014 (Tenhagen et al. 2014; Tenhagen et al. 2018). In previous studies, up to 60% of dairy cows within herds carried MRSA (Spohr et al. 2011; Locatelli et al. 2017; Falk 2018). In our study, all farms had a history of MRSA detection (publication 2). MRSA were detected in milk samples (BTM and/or QMS) from 14/20 farms. The positive test rate in QMS was up to 43% within farms. In summary, the overall MRSA burden in dairy herds is difficult to evaluate and varies widely between studies and regions. In single herds, MRSA might become a severe udder health problem.

For the development of MRSA prevention and control recommendations, it is necessary to identify possible risk factors for the occurrence and transmission of MRSA in dairy herds. According to the literature review, improper milking time hygiene may contribute to the spread of MRSA in dairy herds (Antoci et al. 2013; Guimaraes et al. 2017; Locatelli et al. 2017). In addition, several studies provide evidence that housing pigs close to dairy farms increases the risk for the occurrence of MRSA in dairy herds (Tavakol et al. 2012; Feltrin et al. 2016). MRSA may also be transferred between humans and cattle (Juhasz-Kaszanyitzky et al. 2007). Beside livestock associated (LA-) MRSA, human-adapted MRSA strains (e.g., ST1 and ST5) were detected in milk samples from dairy cows indicating a possible reverse zoonotic transmission from humans to dairy cows (Pilla et al. 2012; Haenni et al. 2014; Abraham et al. 2017). In a German study, conventional dairy farms were more often affected by MRSA compared to

organic farms (Tenhagen et al. 2018). A positive association between herd size and MRSA prevalence was detected in two studies (Cortimiglia et al. 2016; Tenhagen et al. 2018). Methicillin resistant non-*aureus* staphylococci (MR-NAS) were repeatedly detected in milk of dairy cows (Seixas et al. 2014; de Jong et al. 2018; Qu et al. 2018). Since the *mecA* gene may be transferred between staphylococci, the occurrence of MR-NAS was considered a risk factor for the development of new MRSA strains. Finally, the impact of antibiotic therapy on the occurrence of MRSA was discussed (publication 1). A higher antibiotic treatment frequency was associated with higher numbers of oxacillin resistant *S. aureus* in a study from dairy farms in Thailand (Suriyasathaporn et al. 2012). However, *S. aureus* isolates were only tested for phenotypic oxacillin resistance and not for the *mecA* or *mecC* gene. A study from Canada reported that antimicrobial resistance in non-*aureus* staphylococci was not associated with intramammary antimicrobial use (Nobrega et al. 2018a). On the whole, there is no debate that antibiotic selection pressure is associated with antimicrobial resistance (Chantziaras et al. 2014). However, studies that investigated the proportion of antibiotic resistant bacteria among mastitis causing pathogens from dairy cows did not show increasing levels of resistance over time (Oliver et al. 2011). This is surprising since intramammary antibiotic therapy in dairy cows has been used for decades. A possible explanation for low resistance levels might be that the overall number of bacteria is low in the udder. Some studies considered the healthy udder as a potentially sterile compartment (Rainard 2017). Therefore, less bacteria are exposed to antibiotic selection pressure compared to the intestines and other compartments like the skin and mucous membranes (Lam et al. 2014).

To analyze the occurrence and distribution of MRSA in different habitats on dairy farms, a field study was conducted (publication 2). Samples from different age groups of cattle, humans and the environment of dairy farms were included. The highest proportion of MRSA positive samples was detected in nasal swabs of milk-fed calves (22.7% (46/201; 95% CI:17.1-29.0%)). With increasing age, the MRSA positive test rate was decreasing in the nasal cavities of post-weaning calves and prefresh heifers. Positive test rate was 9.1% (17/187; 95% CI: 5.4-14.2%) in nasal swabs from post-weaning calves and 8.9% (17/191; 95% CI: 5.3-13.9%) in nasal swabs from prefresh heifers. It was hypothesized, that a shift in nasal microbiota composition with increasing age as well as changes in the immune system of calves may contribute to the decreasing MRSA colonization (Chase et al. 2008; Holman et al. 2015). A collection of nasal swabs from dairy cows was not performed because of limited access to the cows' head in many milking parlours. Therefore, a comparison of nasal MRSA colonisation between dairy cows and young stock is not possible in this thesis. In previous studies, MRSA were rarely detected in nasal swabs from dairy calves (Fessler et al. 2012; Spohr et al. 2011). To the best of our knowledge, no study investigated the occurrence of MRSA in samples from prefresh heifers before. In the field study, 2.9% (67/2347; 95%CI: 2.2-3.6%) of QMS from dairy cows

carried MRSA and 7.9% (47/597; 95% CI: 5.5-10.3%) of the dairy cows carried MRSA in one or multiple quarters (publication 2). In a study from Sweden, MRSA were detected in 0.05% (4/8757) of QMS (Unnerstad et al. 2013). A low MRSA prevalence in QMS was also reported from Korea (0.18% (15/9055)) (Kwon et al. 2005). Higher MRSA positive test rates in QMS from dairy cows were reported from India (14.3% (57/400)) and China (8.6% (16/186)) (Shrivastava 2018; Dan et al. 2018). However, comparison between studies is difficult since farms from this study were selected based on previous MRSA reports. Moreover, MRSA enrichment and detection methods differ between studies. In this study a selective two-step enrichment method was used for MRSA detection (Nemeghaire et al. 2014; EFSA 2007). In conventional mastitis laboratories a selective enrichment is usually not performed. Therefore, MRSA detection sensitivity from this study might be higher compared to studies that used standard bacteriological methods. On the other hand, most prevalence studies were focused on mastitis milk samples from dairy cows. In this study, QMS from presumably healthy cows were included as well (publication 2).

All MRSA isolates from the field study (n=190) belong to the LA-MRSA sequence type (ST) 398 (publication 2). This finding is in accordance with previous studies from Europe where ST398 was the most common genotype as well (Cortimiglia et al. 2016; Tenhagen et al. 2018; Kadlec et al. 2019). In Germany, other sequence types (e.g. ST1, ST9, ST22 and ST130) were sporadically detected in milk samples from dairy cows (Schlotter et al. 2014; Tenhagen et al. 2018). In Italy, ST97 seems to be a major MRSA strain in dairy herds beside ST398 (Luini et al. 2015; Feltrin et al. 2016; Locatelli et al. 2016). Worldwide, the diversity of MRSA sequence types from dairy herds was higher with up to eight different MRSA sequence types in a study from China (McKay 2008; Hata 2016; Dan et al. 2018). Phenotypic and genotypic analysis of 33 MRSA strains from this study showed that 28 strains were multi-drug resistant to at least three classes of antimicrobials (publication 4). Beside *mecA* gene mediated β-lactam resistance, MRSA were commonly resistant to tetracycline (*tet*(K) or *tet*(M)), pleuromutilins (*vga*(A) or *vga*(E)) and trimethoprim (*dfr*G or *dfr*K). Similar results were reported from Italy, were the majority of LA-MRSA from BTM and farm workers of dairy farms were considered multidrug resistant (Tomao et al. 2020). In a recent study that was performed in a German mastitis laboratory, only 3/10 LA-MRSA ST398 from dairy cows were multi-drug resistant to at least three classes of antimicrobials (Kadlec et al. 2019). In another German study, 13/27 MRSA ST398 were multi-drug resistant which is also lower compared to the results from this study (Fessler et al. 2010).

4.2 The transmission of MRSA within and between dairy farms

Improper milking time hygiene is a common risk factor for the spread of contagious *S. aureus* mastitis pathogens (Keefe 2012, Graber 2020). In the field study from this thesis (questionnaire and on-farm observations during the milking process), some sort of improper milking time hygiene was observed on all farms with MRSA detection in milk (publication 2). Additionally, MRSA were detected in teat liners on 25% (5/20) of the study farms (publication 2). Therefore, it seems likely that a poor milking time hygiene contributes to the spread of MRSA within farms. MRSA transmission to calves may occur by feeding MRSA contaminated milk (Spohr et al. 2011; Ricci et al. 2017). According to the questionnaire, waste milk feeding was common practice on 16/20 farms from this study (publication 2). However, different other routes of transmission such as farm workers, dust, colostrum feeding, or direct contact during/after parturition may also play a role in MRSA transmission to calves. MRSA detection in suckers from automatic calf feeders on 22.2% (2/9) of the farms that used automatic feeders provides evidence that MRSA may easily spread within groups of calves that share the same sucker. In prefresh heifers and milk samples of dairy cows, similar MRSA *spa*-types were detected, indicating that replacement heifers may exchange MRSA with dairy cows. Seven farmers reported in the questionnaire that they purchased replacement heifers in the last six months before our visit (publication 2).

Because of the high LA-MRSA prevalence in the European pig production, it has been suggested that pigs may serve as a reservoir of MRSA infections for other animal species including dairy cows (EFSA 2009). In previous studies, pig holdings that were located close to dairy farms, increased the risk for the occurrence of MRSA in dairy cows (Spohr et al. 2011; Tavakol et al. 2012; Locatelli et al. 2016). In this study, five farms kept both pigs and cattle on the same farm (publication 2). MRSA were detected in dust samples from two pig barns. On one of them, MRSA staphylococcal cassette chromosome *mec* (SCC*mec)* types and staphylococcal protein A (*spa*) types from pigs and dairy cows were different, indicating that a recent transmission seems unlikely. Therefore, it could not be confirmed that pig holdings are a major risk factor for the occurrence of MRSA on dairy farms from this study as it was previously suggested (publication 1 and 2).

Although, nasal swabs from humans were obtained from only seven farms in this study, MRSA detection in 6/14 human samples (42.9%) suggests that LA-MRSA transmission between humans and cattle occurs (publication 2). A possible LA-MRSA transmission between humans and cattle was previously described (Locatelli et al. 2017; Barberio et al. 2019). The direction of transmission remains unclear in all studies. In the literature review, several studies were identified in which community associated (CA-) and healthcare associated (HA-) MRSA genotypes were detected in samples from dairy cows (Pilla et al. 2012; Alba et al. 2015;

Abraham et al. 2017). It was concluded that reverse zoonotic transmission of MRSA from humans to dairy cows is also possible. Since all isolates from this study belong to the LA-MRSA ST398 the transmission of CA- and HA-MRSA between humans and cattle cannot be confirmed.

Finally, similar LA-MRSA *spa*-types were detected in milk samples, young stock, humans and the environment on 13/17 MRSA affected farms (publication 2). A transmission of the same LA-MRSA strain within the farms seems likely. These findings underline the ability of MRSA ST398 to colonize various age groups of cattle, humans and the environment on dairy farms. Transmission of LA-MRSA to dairy cows may occur during the milking process and via MRSA affected replacement animals. Environmental vectors such as dust (wind), other animal species, farm workers and people who visit different farms (e.g. veterinarians and breeding-technicians) may also contribute to LA-MRSA transmission within and between dairy farms.

4.3 MRSA and udder health

As described in the literature review, the impact of MRSA on udder health of dairy cows differs widely between studies (publication 1). Somatic cell count measurements from QMS in this study provide evidence that MRSA can cause mastitis in dairy cows (publication 2). The geometric mean somatic cell count of MRSA affected QMS from this study was 345,000 cells/ml and in all QMS included in the study the somatic cell count was 114,000 cells/ml. QMS with high somatic cell counts (>150,000 cells/ml in primiparous cows and >250,000 cells/ml in multiparous cows) were six times more likely to carry MRSA compared to QMS with somatic cell counts below the threshold (OR=6.153, $p<0.001$). In addition, most farms with MRSA detection in QMS showed elevated BTM somatic cell counts (>250,000 cells/ml) in the last three months before our visit (publication 2). High BTM somatic cell counts have been considered a main indicator of *S. aureus* affected dairy herds and the same applies to MRSA affected herds from this study (NMC 2017; Blowey and Edmondson 2010). Since milk samples from our study were selectively tested for MRS, the contribution of other pathogens to elevated somatic cell counts cannot be excluded. Results from whole genome sequencing of 33 MRSA strains showed multiple virulence factor associated genes harboured by the MRSA strains. Frequently detected virulence genes were the clumping factor encoding genes "*clfA"* and "*clfB"*, the collagen adhesion protein gene "*can"*, the fibronectin-binding protein gene "*fnbA"* and genes that encode biofilm formation (*icaA*, *icaB*, *icaC*, *icaD* and *icaR*). It was concluded that the virulence factors may be associated with mastitis pathogenesis in dairy cows (publication 4). However, the pathogenesis of *S. aureus* as a mastitis causing pathogen is complex and not fully understood (Kerro Dego et al. 2002; Magro et al. 2017; Naushad et al. 2020). In a recent study, whole genome sequencing of 119 bovine *S. aureus* strains was

performed (Naushad et al. 2020). No association between the number of virulence factor associated genes and severity of mastitis (somatic cell counts in milk) was detected. The authors concluded that the presence of certain virulence factors does not allow a prediction of *S. aureus* pathogenicity in the cows' udder.

4.4 MRSA monitoring, prevention and control

Previous studies suggest that MRSA prevalence in dairy herds might be increasing in some countries (Tenhagen et al. 2014; Tenhagen et al. 2018; Song et al. 2016). Results from this study provide evidence, that MRSA may not only colonize the cows' udder but also young stock, humans and the environment on dairy farms (publication 2). Therefore, MRSA monitoring in dairy herds should be continued.

In the field study, MRSA were detected on 17/20 dairy farms (publication 2). On 10/12 farms, MRSA were detected in QMS and in BTM. On two more farms, MRSA were detected in BTM but not in QMS from preselected cows. Therefore, BTM screening seems to be useful to predict the occurrence of MRSA in milk of dairy cows. This is important since the implementation of BTM sampling is easy to achieve compared to the collection and analysis of QMS. To further improve the MRSA detection rate on dairy farms, nasal swabs from milk-fed calves and BTM samples could be analyzed in parallel testing. Combining both sample types (BTM and swab samples from milk-fed calves), all MRSA positive dairy farms from this study (17/17) would have been identified (publication 2).

Since MRSA may cause mastitis in dairy cows and pose a risk for human health, strategies are needed to reduce the emergence and spread of MRSA in dairy herds. For MRSA prevention and control on dairy farms, the following recommendations can be made according to the results of this thesis:

I. Maintenance of proper milking time hygiene procedures to prevent the spread of contagious mastitis (Keefe 2012; Edmondson 2020; publication 2).
    - Use of 1 clean udder cloth for drying and cleaning teats
    - Use of clean gloves during milking
    - Application of post-dipping teat disinfectants
    - Milking infected cows last
    - Perform cluster disinfection after every milking process

II. If replacement animals are purchased, they should be tested for MRSA colonization before entering the herd. From prefresh heifers both nasal and udder cleft swabs were collected in this study (publication 2). The individual positive test rates for MRSA detection in both nasal swabs (57.7%) and udder cleft swabs (34.6%) of prefresh

heifers were low. Only 2/26 MRSA positive heifers (7.7%) carried MRSA in their nasal cavities and in the udder cleft. Therefore, if heifers are tested for MRSA colonization, multiple body sites should be included in parallel testing (publication 2).

III. To reduce the MRSA transmission from dairy cows to calves, waste milk feeding should be avoided. Alternatively, waste milk and colostrum should be heat treated to reduce the risk of MRSA transmission via contaminated milk (publication 2). If colostrum is heat treated before feeding, it is important to follow the temperature and heating time recommendations to maintain sufficient immunoglobulin concentrations (McMartin et al. 2006; Heinrichs 2017).

IV. Since LA-MRSA ST398 may colonize various animal species, contact with other animals should be avoided (publication 2). If people work with different animal species on the same farm, washing hands, changing clothes and cleaning equipment before changing between species is recommended.

V. LA-MRSA also colonize humans. Therefore, barriers for external people on dairy farms are recommended. People who visit many farms per day (e.g. veterinarians and breeding-technicians) should change clothes, clean boots and wash hands before entering the dairy barn and before changing between groups of animals. Farm workers should also be aware of biosecurity measures like washing hands and changing clothes regularly.

Treatment of MRSA infections is challenging since most antibiotics approved for mastitis therapy in dairy cows are β-lactams (VETIDATA 2018). Especially cloxacillin has been widely recommended for treatment of *S. aureus* infections during the dry period (Makovec and Ruegg 2003; Tenhagen et al. 2006; Saini et al. 2012b). Treatment with cloxacillin and other semisynthetic penicillins is probably ineffective to cure MRSA infections. Therefore, culling of infected animals has been recommended (Spohr et al. 2011). Quickly removing infected cows from the herd may be the only way to hinder the spread of MRSA on dairy farms. On the other hand, results from this thesis show that MRSA are widespread among different age groups of cattle and in the environment of dairy farms (publication 2). Therefore, MRSA may be repeatedly introduced into the dairy cow herd and the MRSA eradication may not be achieved by culling infected cows alone. The effectiveness of non-β-lactam antimicrobials (e.g., pirlimycin) to treat MRSA infections in dairy cows should be investigated in the future. Since pirlimycin therapy is time-consuming and *S. aureus* cure rates are low, independent of the therapeutic substance, treatments might be an option for single dairy cows like newly infected primiparous cows. Alternative treatment and prevention measures like vaccines, phage-therapy and bacteriocins may be used in the future. To date, the success of *S. aureus* vaccines

is limited, and effectiveness was not investigated for LA-MRSA ST398 (Schukken et al. 2014; NMC 2017). Bacteriophages were shown to lyse bovine *S. aureus* strains in vitro (Dias et al. 2013; Li and Zhang 2014; Titze et al. 2020). However, in vivo treatment is challenging. The same applies to bacteriocins like nisin and lacticin which may be used for treatment of MRSA infections after further in vivo research and successful medical approval (Carson et al. 2017; Castelani et al. 2019).

4.5 Implications for human health

In 22/67 MRSA positive QMS from this study, somatic cell counts were low (<150,000 cells/ml in primiparous cows and <250,000 cells/ml in multiparous cows). MRSA contaminated milk from such clinically healthy cows may enter the bulk tank. MRSA were repeatedly detected in BTM from different studies (publication 1; Tenhagen et al. 2018). Thus, MRSA might enter the dairy food chain and can be transferred to consumers of raw milk and raw milk products. Raw milk consumption is common among dairy farmers and milk has been sold to consumers by raw milk vending machines (Heimann 2020). It remains unclear if the consumption of MRSA contaminated milk and dairy products may cause MRSA colonization in humans. Nasal MRSA colonization was observed in calves that received MRSA contaminated milk (Spohr et al. 2011). Among 33 LA-MRSA isolates from this study, no staphylococcal enterotoxin genes were detected, indicating a low risk for food intoxications caused by the LA-MRSA strains from this study (publication 4). Only few studies have detected enterotoxin genes in LA-MRSA ST398 before (Kadlec et al. 2009; Wendland et al. 2013).

The proportion of clinical MRSA infections in humans caused by LA-MRSA ST398 is rather low in Germany (approximately 4%) (Layer et al. 2019). However, in areas with high livestock density (e.g. Münsterland), the proportion of LA-MRSA ST398 infections among human patients was up to 35% (Cuny et al. 2015, Van Alen et al. 2017). Detection of similar MRSA strains in samples from humans and cattle from this study underlines the possible transmission of LA-MRSA between cattle and humans on dairy farms (publication 2). The risk for severe infections in humans caused by LA-MRSA strains from this study seems to be low (publication 4). Whole genome sequencing analysis of 33 MRSA isolates from this study revealed low numbers of human associated virulence factor associated genes in the MRSA strains (publication 4). The Panton-Valentine leukocidin (*pvl)* gene, the toxic shock syndrome toxin (*tsst)* gene and genes of the human associated immune evasion cluster (IEC) were not detected in LA-MRSA ST398 from this study. This is in line with previous studies in which human associated virulence genes were rarely detected in LA-MRSA ST398 (Ballhausen et al. 2017; Argudin et al. 2011). However, LA-MRSA ST398 sporadically caused severe infections in humans like wound infections and even septicaemia (Goerge et al. 2017).

Therefore, people working on dairy farms should be aware of possible MRSA colonization. Especially injuries and areas of broken skin should be covered with a waterproof dressing to avoid MRSA entering the body. In addition exposed people should be tested for MRSA colonization before surgical procedures and hospital admittance (Cuny et al. 2015).

4.6 MR-NAS in dairy herds

Besides MRSA, we also detected MR-NAS species in the framework of this thesis (publication 3). Comparing MR-NAS and MRSA positive test rates, MR-NAS were more frequently detected in all sample types except for BTM. This indicates that MR-NAS are more prevalent on the study farms than MRSA. The geometric mean somatic cell count in MR-NAS affected QMS was 183,000 cells/ml and MR-NAS affected QMS were approximately two times more likely to have high somatic cell counts (>150,000 cells/ml in primiparous cows and >250,000 cells/ml in multiparous cows) compared to all QMS from this study (OR=1.838; p=0.019). Consequently, MR-NAS from this study may have a negative impact on udder health. Since only MRS were detected in this study, the contribution of other pathogens to elevated somatic cell counts cannot be excluded. Compared to the somatic cell count in MRSA affected QMS (345,000 cells/ml), the impact of MR-NAS on udder health is low. This is in line with previous studies that considered NAS as mastitis causing pathogens of minor importance for udder health compared to *S. aureus* as a major pathogen (Blowey and Edmondson 2010). In a recent study from Belgium, the *mecA* gene was detected in 49% of NAS from clinical mastitis compared to 6% *mecA* positive NAS from healthy quarters (Wuytack et al. 2020a). It was concluded that MR-NAS might be more pathogenic compared to methicillin susceptible NAS. Since only MR-NAS were detected in this study, a comparison with methicillin susceptible NAS and their impact on udder health is not possible.

Bacterial culture and molecular analysis of MR-NAS is also important since resistance genes may be transferred from MR-NAS to *S. aureus*, leading to higher numbers of MRSA (Miragaia 2018). The transfer of the *mecA* harbouring gene cassette (SCC*mec*) between staphylococcal species has been observed in vitro (Morikawa et al. 2012; Chlebowicz et al. 2014; Ray et al. 2016). In this study, SCC*mec* type V was detected in MR-*S. epidermidis*, MR-*S. cohnii* and MRSA on one farm (publication 3). MR-*S. haemolyticus* isolates from six farms carried SCC*mec* type V as the MRSA on the corresponding farms. It might be possible that the SCC*mec* cassette was transferred in any direction between *S. aureus*/MRSA and NAS/MR-NAS on these study farms. In addition, a transfer of the *mecA* gene independent of the SCC*mec* was suggested (Fisher and Paterson 2020).

The most frequently detected MR-NAS species in this thesis was MR-*S. sciuri* which was detected in samples from dairy cows, young stock, and the environment (publication 3). MR-

S. sciuri was the most common MR-NAS species in previous studies from dairy farms as well (Frey et al. 2013; Mahato et al. 2017; Fischer and Paterson 2020). In some studies, MR-*S. epidermidis* was the most frequently detected MR-NAS isolate in milk samples of dairy cows (Taponen et al. 2016; Nobrega et al. 2018b; Kim et al. 2019). In this study, MR-*S. epidermidis* was detected in only five QMS. Since MR-*S. epidermidis* were shown to exhibit low levels of phenotypic oxacillin/cefoxitin resistance, the lower detection rate in this study might be caused by the cefoxitin based selective enrichment method used for bacterial culture in this study (publication 3). Additionally, 138 *S. cohnii* isolates were detected, that showed a reduced susceptibility to at least 4 mg/l cefoxitin but did not carry the *mecA* or *mecC* gene. Therefore, cefoxitin susceptibility testing is not reliable to predict the *mecA*/*mecC* gene mediated methicillin resistance in *S. cohnii* isolates from dairy cows in this study. The genetic background of cefoxitin resistance in the *S. cohnii* isolates from this study should be investigated in the future using whole genome sequencing analysis.

The most frequently detected MR-NAS species from clinical mastitis worldwide is *S. chromogenes* (Condas et al. 2017; Vanderhaeghen et al. 2015*).* In this study no MR-*S. chromogenes* was detected (publication 3). In previous studies, *S. chromogenes* rarely harboured the *mecA* gene (Cicconi-Hogan et al. 2011; Seixas et al. 2014). The reason for high proportions of *S. chromogenes* isolates among NAS from mastitis milk samples remains unclear. Broad spectrum β-lactam resistance is probably not the main driver of *S. chromogenes* spread in dairy herds.

4.7 Limitations

Since the prevalence of MRSA was reported to be low in dairy herds (publication 1), we selected dairy farms based on previous MRSA reports to increase the probability for MRSA positive test results (publication 2 and 3). Therefore, this was not a typical cross-sectional study and the results do not represent all German dairy farms. The positive test rates give an overview on the distribution of MRSA and MR-NAS within preselected farms.

In the laboratory, a two-step enrichment method (2-S method) using Mueller-Hinton broth with 6% NaCl and tryptic soy broth supplemented with cefoxitin (3.5 mg/l) and aztreonam (50 mg/l) was performed. After the enrichment, bacteria were cultured on cefoxitin (4 mg/l) agar plates (publication 2 and 3). This method has been recommended for MRSA detection by the European Food Safety Authority (EFSA 2007). Recent publications suggest that skipping the second enrichment step (1-S method) increases LA-MRSA ST398 detection sensitivity in samples from pigs and chicken meat (Larsen et al. 2017; Pauly et al. 2019). In samples from cattle, no difference was observed between the 2-S method used in this study and the 1-S method regarding LA-MRSA detection sensitivity (Nemeghaire et al. 2014). Further research

is needed to validate MRSA detection sensitivity in samples from cattle using the 1-S and 2-S method.

For NAS as a group of potential pathogens, standardized recommendations for phenotypic methicillin resistance testing are not available. For the *mecA*/*mecC* gene prediction in some NAS species (*S. pseudintermedius* and *S. scheiferi*), oxacillin was shown to be more reliable compared to cefoxitin (Swenson and Tenover 2005; EUCAST 2020). Furthermore, some studies reported oxacillin susceptible *mecA*-positive NAS species (Mahato et al. 2017). Therefore, MR-NAS detection rates from this thesis might be underestimated using relatively high cefoxitin concentrations for bacterial culture (publication 3).

According to recent phylogenomic investigations, NAS from the *S. sciuri* group were reassigned to the novel genus *Mammaliicoccus* within the family *Staphylococcocaceae* (Madhaiyan et al. 2020). Therefore, the species *S. sciuri*, *S. lentus*, *S. fleurettii* and *S. vitulinus* will be referred to as '*Mammaliicoccus*' instead of '*Staphylococcus*' in the future.

5 Summary

Methicillin resistant staphylococci on German dairy farms - Aspects of distribution, transmission, and control on farm level

Methicillin resistant staphylococci (MRS) can cause mastitis in dairy cows and zoonotic infections in humans. Due to limited treatment options, mastitis caused by MRS is a severe animal health problem. While MRS were repeatedly detected in milk samples, there is a lack of knowledge regarding other MRS habitats and possible routes of transmission on dairy farms. Therefore, this thesis aimed to investigate aspects of MRS distribution, transmission and control on dairy cattle farms.

A literature review was performed to identify possible risk factors for the occurrence of methicillin resistant *Staphylococcus* (*S.*) *aureus* (MRSA) on dairy farms. Factors that might increase the risk for the occurrence of MRSA in dairy herds are 1) improper milking time hygiene, 2) pig holdings close to dairy farms, 3) MRSA affected humans on dairy farms 4) methicillin resistant non-*aureus* staphylococci (MR-NAS), 5) a larger herd size and 6) a conventional production system.

In a field study, samples from dairy cows, young stock, humans and the environment of dairy farms were collected for bacterial culture (n=3167). Study farms (n=20) were selected based on MRSA reports from previous years. General farm management data and milking time hygiene procedures were analyzed using a questionnaire. All MRSA isolates from the field study (n=190) were assigned to the livestock-associated MRSA sequence type (ST) 398. The staphylococcal cassette chromosome *mec* (SCC*mec*) type V was most frequently detected among MRSA isolates. The most common staphylococcal protein A (*spa*-) types (t) were t011 and t034. Further analysis of 33 MRSA isolates showed multi-drug resistant phenotypes and related genes in most isolates.

MRSA were detected in 2.9% (67/2347) of QMS and 7.9% (47/597) of dairy cows carried MRSA in one or multiple quarters. The geometric mean somatic cell count in QMS was higher in MRSA affected quarters (345,000 cells/ml) compared to all QMS (114,000 cells/ml) indicating a significant impact of MRSA on udder health. All farms with MRSA detection in milk were not consistently following milking time hygiene procedures to prevent contagious mastitis. The highest MRSA positive test rate was 22.7% (46/203) in nasal swabs from milk-fed calves. In post-weaning calves, 9.1% (17/187) of nasal swab samples carried MRSA. In nasal swabs from prefresh heifers, MRSA positive test rate was 8.9% (17/191) and in udder cleft swabs from prefresh heifers positive test rate was 6.5% (11/170). It was concluded that young stock carries high loads of MRSA that can be transferred to dairy cows. In this study, five farms kept both pigs and cattle, and MRSA were detected in dust samples from two pig barns. On one

farm, MRSA genotypes were identical and on the other farm, different isolates were detected in samples from the pig barn and from the dairy barn. Thus, pigs are probably not a major reservoir of MRSA infection for dairy cows on the study farms. MRSA were detected in 42.9% (6/14) of human nasal swabs and MRSA genotypes were similar to the cattle strains on the corresponding farms. Therefore, transmission between the species seems likely and MRSA in cattle may pose a risk for humans working on dairy farms. In the environment, MRSA were detected in dust samples from five farms, in teat liners from five farms and in two automatic calf feeders. On 13/20 farms, similar MRSA genotypes were detected in samples from dairy cows (QMS and/or bulk tank milk) and in samples from young stock or the environment. Similar MRSA isolates were detected in all groups of young stock (milk-fed calves, post-weaning calves and prefresh heifers) on four farms.

MR-NAS were detected on all dairy farms and positive test rates were usually higher compared to MRSA positive test rates. The most common MR-NAS species where *S. sciuri*, *S. lentus*, *S. fleurettii*, *S. epidermidis* and *S. haemolyticus*. In addition, high numbers of *S. cohnii* isolates were detected that showed a reduced susceptibility to cefoxitin but did not carry the *mecA* or *mecC* gene. Somatic cell counts in MR-NAS affected QMS (183,000 cells/ml) were higher compared to all QMS from this study (114,000 cells/ml) indicating a slight but significant impact of MR-NAS on udder health. MR-NAS may pose a risk for the development of new MRSA strains since resistance genes can be transferred between staphylococcal species.

In conclusion, MRS not only affect the cows' udder. MRS frequently spread among different age groups of cattle, humans, and the environment of dairy farms. This thesis stresses the need for continuous MRS monitoring and identification of unknown MRS habitats on dairy farms. The results may serve as a basis for MRS monitoring and control strategies in the future.

6 Zusammenfassung

Methicillin resistente Staphylokokken in deutschen Milchviehbetrieben - Aspekte der Verbreitung, Übertragung und Kontrolle auf Betriebsebene

Methicillin resistente Staphylokokken (MRS) sind potentielle Zoonose Erreger und können Mastitis beim Rind verursachen. Infektionen des Euters mit MRS sind problematisch, da in der Tiermedizin kaum wirksame Behandlungsoptionen für beta-Laktam resistente Mastitiserreger bestehen. Während MRS häufig in Milchproben nachgewiesen wurden, ist wenig über das Vorkommen in anderen Habitaten und über mögliche Verbreitungswege auf Milchviehbetrieben bekannt. Daher war es das Ziel dieser Arbeit, das Vorkommen sowie Aspekte der Übertragung und Kontrolle von MRS in Milchviehbetrieben zu untersuchen.

Eine Literaturanalyse wurde durchgeführt, um mögliche Risikofaktoren für das Vorkommen von Methicillin resistenten *Staphylococcus* (*S.*) *aureus* (MRSA) in Milchviehherden zu identifizieren. Faktoren, die das Risiko für MRSA im Milchviehbetrieb erhöhen könnten, sind 1) mangelhafte Melkhygiene, 2) Schweinehaltungen in der Nähe von Milchviehställen, 3) mit MRSA kolonisierte Menschen, die Kontakt zu Rindern haben, 4) das Vorkommen von Methicillin resistenten nicht-*aureus* Staphylokokken (MR-NAS) 5) eine größere Herde und 6) ein konventionelles Produktionssystem.

Im Rahmen einer Feldstudie wurden Proben von Milchkühen, Jungtieren, Menschen und aus der Umwelt von Milchviehbetrieben gesammelt (n=3167). In den 20 Studienbetrieben wurden bereits in der Vergangenheit MRSA in Milchproben nachgewiesen. Allgemeine Betriebsdaten sowie Maßnahmen der Melkhygiene wurden mit Hilfe eines Fragebogens dokumentiert.

Alle MRSA Isolate aus der Feldstudie (n=190) gehörten zum nutztierassoziierten Sequenz Typ (ST) 398 und trugen das *mecA* Gen. Die Typisierung der *mec*-Genkassette (SCC*mec* Typ) ergab, dass bis auf zwei Ausnahmen (IVa) alle MRSA den SCC*mec* Typ V trugen. Die Typisierung des *S. aureus* spezifischen Protein A (*spa*-Typ) ergab, dass die meisten Isolate den Typen t034 und t011 zugeordnet werden konnten.

MRSA wurde in 2,9% (67/2347) der Viertelgemelksproben (VGP) nachgewiesen und 7,9% (47/597) der Milchkühe waren betroffen. Die Zellzahl (geometrische Mittel) war höher in MRSA positiven VGP (345.000 Zellen/ml) im Vergleich zu allen VGP (114.000 Zellen/ml). Dies deutet daraufhin, dass MRSA Mastitis verursachen. In allen Betrieben mit MRSA Befunden aus Milchproben wurden die Melkhygiene-Empfehlungen zur Vermeidung kontagiöser Mastitis Erreger nicht konsequent eingehalten.

Die insgesamt höchste MRSA Nachweisrate wurde in Nasentupfern von Milchkälbern detektiert (22,7% (46/203)). In Nasentupfern von abgesetzten Kälbern lag die Nachweisrate bei 9,1% (17/187). In Nasentupfern von tragenden Färsen lag die Nachweisrate bei 8,9%

(17/191) und in Schenkelspalttupfern der Färsen bei 6,5% (11/170). Somit sind Jungtiere aus dieser Studie häufig mit MRSA besiedelt und die Bakterien könnten auf die Milchkühe übertragen werden. Da Schweine von allen Nutztieren am häufigsten mit MRSA ST398 besiedelt sind, wird vermutet, dass Schweine ein Risiko für den MRSA Eintrag in Kuhställe darstellen. Fünf Betriebe dieser Studie hielten neben Kühen auch Schweine. In zwei Betrieben wurden MRSA im Kuhstall und in Staubproben aus Schweineställen desselben Standortes detektiert. Auf einem der Betriebe waren die MRSA Genotypen aus Kuh- und Schweinestall identische, während die Isolate auf dem anderen Betrieb unterschiedlich waren. In Betrieben dieser Studie stellen Schweine also wahrscheinlich kein bedeutendes Risiko für den MRSA Eintrag in Milchviehherden dar. MRSA wurden außerdem in 42,9% (6/14) der Nasentupferproben vom Betriebspersonal nachgewiesen und identische MRSA Genotypen wurden auch in Proben von Rindern auf den jeweiligen Betrieben detektiert. Es ist also wahrscheinlich, dass MRSA ST398 zwischen Menschen und Rindern übertragen werden. Auf fünf Betrieben wurden MRSA in Staubproben aus dem Kuhstall und in Tupferproben aus Zitzenbechern nachgewiesen. Insgesamt wurden auf 13/20 Betrieben die gleichen MRSA Genotypen in Proben von Milchkühen (VGP und/oder Tankmilch) und in Proben von Jungtieren oder der Umwelt nachgewiesen. Auf vier Betrieben wurden außerdem die gleichen MRSA Typen in allen Jungtiergruppen (Milchkälber, abgesetzte Kälber und tragende Färsen) detektiert.

MR-NAS wurden in Proben von allen Milchviehbetrieben nachgewiesen und MR-NAS wurden in der Regel häufiger detektiert als MRSA. Die häufigsten MR-NAS Spezies waren *S. sciuri*, *S. lentus*, *S. fleurettii*, *S. epidermidis* und *S. haemolyticus.* Außerdem wurden zahlreiche *S. cohnii* Isolate detektiert, die phänotypisch Cefoxitin resistent waren, allerdings kein *mecA* oder *mecC* Gen trugen. Die Zellzahl (geometrisches Mittel) in VGP mit MR-NAS Nachweis (183,000 Zellen/ml) war höher im Vergleich zum Zellzahldurchschnitt aller VGP (114,000 Zellen/ml). MR-NAS wirken sich somit negativ auf die Eutergesundheit aus, wobei der Effekt geringer ist als in MRSA betroffenen Euterviertel. MR-NAS können außerdem Resistenzgene mit *S. aureus* austauschen und somit zur Entstehung neuer MRSA beitragen.

Abschließend lässt sich sagen, dass das Vorkommen von MRS nicht auf die Milch und damit die Euter der Kühe beschränkt ist. MRS sind bei unterschiedlichen Altersgruppen von Rindern, beim Menschen und in der Umwelt von betroffenen Milchviehbetrieben verbreitet. Diese Arbeit zeigt, wie wichtig es ist, das Vorkommen von MRS in Milchviehherden auch in Zukunft weiter zu untersuchen und mögliche unbekannte Erregernischen zu identifizieren. Neue Erkenntnisse können außerdem dazu beitragen Monitoring- und Präventionsstrategien für MRS in Milchviehbetrieben zu entwickeln.

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8 List of Publications

Journal articles:

Schnitt, A. and B. A. Tenhagen. (2020):
Risk Factors for the Occurrence of Methicillin-Resistant *Staphylococcus aureus* in Dairy Herds: An Update.
Foodborne Pathog. Dis. 17(10): 585-596

Schnitt, A., T. Lienen, H. Wichmann-Schauer, C. Cuny, and B. A. Tenhagen. (2020):
The occurrence and distribution of livestock-associated methicillin-resistant *Staphylococcus aureus* ST398 on German dairy farms.
J. Dairy Sci. 103 (12): 11806-11819

Lienen, T., A. Schnitt, J. A. Hammerl, S. Maurischat, and B.-A. Tenhagen. (2021):
Genomic Distinctions of LA-MRSA ST398 on Dairy Farms From Different German Federal States With a Low Risk of Severe Human Infections.
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Schnitt, A., T. Lienen, H. Wichmann-Schauer and B. A. Tenhagen. (2021):
The occurence of methicillin resistant non-*aureus* staphylococci in samples from cows, young stock and the environment of German dairy farms.
J. Dairy Sci., 104 (4): 4604-4614

Oral presentations:

Schnitt, A., T. Lienen, H. Wichmann-Schauer and B. A. Tenhagen. (2021)
Methicillin resistant non-*aureus* staphylococci on German dairy farms.
National Mastitis Council 60. Annual Meeting: 26.1.-29.1.2020, online conference

Schnitt, A., T. Lienen, H. Wichmann-Schauer and B. A. Tenhagen. (2020)
Vorkommen und Verbreitung von Methicillin resistenten Koagulase negativen Staphylokokken in Milchviehherden.
DVG-Vet-Congress: 15.-17.10.2020, online conference

Schnitt, A., T. Lienen, H. Wichmann-Schauer and B. A. Tenhagen. (2020)
Methicillin Resistant *Staphylococcus aureus* in German Dairy Herds.
National Mastitis Council 59. Annual Meeting: 28.1.-31.1.2020, Orlando, USA

Schnitt, A., T. Lienen, H. Wichmann-Schauer and B. A. Tenhagen. (2019)
Risikofaktoren für das Vorkommen von Methicillin resistenten *Staphylococcus aureus* (MRSA) in deutschen Milchviehherden.
DVG-Vet-Congress: 14.-16.11.2019, Berlin.

Schnitt, A., T. Lienen, H. Wichmann-Schauer and B. A. Tenhagen. (2019)
Methicillin resistant staphylococci in German dairy herds – Involved species and genes.
IDF Mastitis Conference: 14-17.05.2019, Copenhagen, Denmark.

Poster presentations:

Schnitt, A., T. Lienen, H. Wichmann-Schauer and B. A. Tenhagen. (2020)
Methicillin Resistant *Staphylococcus aureus* in German Dairy Herds.
National Mastitis Council 59. Annual Meeting: 28.1.-31.1.2020, Orlando, USA

Schnitt A., T. Lienen, H. Wichmann-Schauer and B.-A. Tenhagen (2020)
Methicillin resistant non-*aureus* staphylococci on German dairy cattle farms.
National Symposium on Zoonoses Research 2020, 15.-16.10.2020, online conference

Schnitt A., T. Lienen, H. Wichmann-Schauer and B.-A. Tenhagen (2019)
Outbreak of mastitis caused by LA-MRSA CC398 in a Bavarian dairy herd.
National Symposium on Zoonoses Research 2019, 16.-18.10.2019, Berlin.

Schnitt A. and B.-A. Tenhagen (2018)
Risk factors for the occurrence of methicillin resistant *Staphylococcus aureus* (MRSA) in dairy herds – an update.
National Symposium on Zoonoses Research 2018, 17.-19.10.2018, Berlin.

9 Acknowledgements

Ich bedanke mich herzlich bei allen Personen aus der Abteilung „Biologische Sicherheit“ des Bundesinstitutes für Risikobewertung, die mich bei der Durchführung dieser Arbeit unterstützt haben. Insbesondere danke ich

PD Dr. Bernd-Alois Tenhagen für die Überlassung des Themas sowie für die konstruktive Diskussion wissenschaftlicher und praktischer Fragestellungen. Außerdem danke ich Herrn Tenhagen für das hohe Maß an Freiheit, das er mir bei der Durchführung und Ausgestaltung dieser Arbeit überlassen hat.

Dr. Tobias Lienen für die fachliche und praktische Unterstützung sowohl bei der Arbeit im Feld wie auch im Labor.

Dr. Heidi Wichmann-Schauer für das organisatorische Mitwirken und die fachliche Unterstützung.

Ich danke außerdem Dr. Christiane Cuny vom Robert Koch Institut für die Untersuchung der Humanproben.

Mein Dank gilt zudem allen Landwirtinnen und Landwirten die freiwillig an meiner Studie teilgenommen haben. Insbesondere für die Geduld beim Melken und für das Teilen betriebsinterner Daten.

Ich danke allen Personen, die mich bei der Rekrutierung der Studienbetriebe unterstützt haben. Hierzu zählen Dr. Ulrike Sorge, Dr. Thomas Peters, Dr. Sabine Reinhold, Oliver Claushues, Dr. Natalie Morgenstern, Dr. Karin Eulenberger und PD Dr. Karsten Donat.

Mein Dank gilt auch Dr. Stefan Borchardt von der Freien Universität Berlin für die Unterstützung bei der Auswertung von Herdenmanagement Daten.

Dem National Mastitis Council (NMC) danke ich für das Reisestipendium, welches mir eine Teilnahme an der 59. Jahrestagung in Orlando ermöglichte.

Ich bedanke mich beim Bundesministerium für Bildung und Forschung (BMBF) für die Finanzierung meiner Promotion im Rahmen des Projektes #1Health-PREVENT, Förderkennzeichen: 01KI1727A.

10 Declaration of Independence

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, 03.09.2021 Max-Arne Schnitt

11 Appendix

Bundesinstitut für Risikobewertung

Fragebogen zu Methicillin resistenten *Staphylococcus aureus* (MRSA) in Milchviehbetrieben

Datum

Anwesende _____

Betrieb (Standorte)

_____

_____

BetriebsleiterIn/HerdenmanagerIn/AnsprechpartnerIn

_____

_____

Bundesinstitut für Risikobewertung

1. Generelle Herdeninformation

Betriebsform ☐ ökologisch ☐ konventionell

Rasse _____

Anzahl Kühe _____

Gruppeneinteilung
- ☐ Frischmelker
- ☐ Hochleistung
- ☐ Altmelker
- ☐ Trockensteher
- ☐ Euterkranke
- ☐ Special needs

Weitere Tierarten im Betrieb
- ☐ Mastschweine
- ☐ Ferkelproduktion
- ☐ Masthähnchen
- ☐ Legehennen
- ☐ Puten
- ☐ Hund/Katze
- ☐ _____
- ☐ _____
- ☐ _____
- ☐ _____

Mitarbeiterverkehr zwischen Tierarten ☐ ja ☐ nein

Weitere Tierhaltungen in Umgebung _____

Personenverkehr
- ☐ Tierarzt/Tierärztin

Kontakt Schweine/Geflügel? ☐ ja ☐ nein

- ☐ BesamerIn
- ☐ ViehhändlerIn
- ☐ FuttermittelberaterIn
- ☐ KlauenschneiderIn
- ☐ AbdeckerIn
- ☐ MilchfahrerIn
- ☐ _____

Weidegang / Außenauslauf ☐ ja ☐ nein

Milchleistung

_____

(Jahresdurchschnitt vorläufige MLP-Daten)

Abgangsrate _____

(Kuhabgänge – Zuchtverkäufe)

davon wegen Mastitis _____

Herkunft der Nachzucht

Anzahl zugekaufter Tiere (12 Mo)_____

Untersuchung
- ☐ S. aureus
- ☐ Strep. agalactiae
- ☐ Mykoplasmen
- ☐ _____

Seit wann ist der MRSA Status bekannt? _____

Dokumentation/ Herdensoftware _____

Verkauf Rohmilch ab Hof/Milchtankstelle _____

Konsum Rohmilch ☐ ja ☐ nein

Humane MRSA Infektionen im Betriebsumfeld ☐ ja ☐ nein

2. Eutergesundheit - Herde

Routineproben
- ☐ Frischabkalber
- ☐ zum Trockenstellen
- ☐ klinische Mastitis
- ☐ Zellzahlkühe

Zellzahl Kühe _____

(MLP Daten - min. letzte 3 Monate)

Zellzahl Färsen _____

(MLP Daten - min. letzte 3 Monate)

Dokumentation (wo & was?) _____

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3. Trockenstellen

Wann und wie lange _____

Trockensteller (Präparate) _____

Selektiv ? ☐ ja ☐ nein
Schulung/SOPs ☐ ja ☐ nein
Zitzenversiegler ☐ ja ☐ nein
Abflämmen ☐ ja ☐ nein

4. Melken Wie oft? ☐ 2x ☐ 3x

Melkreihenfolge _____

Wie viele MelkerInnen _____

Melkstand

Typ & Hersteller _____

Gesamteindruck _____

Atmosphäre _____

Anteil Tiere die Kot/Urin absetzen _____

R&D Intervall, Substanzen _____

_____

Zitzengummis

Wechselperiode _____

Sauber ☐ schmutzig ☐ glatt ☐ angeraut ☐

Melkroutine

Schulung/SOPs ☐ ja ☐ nein
Prädippen ☐ ja ☐ nein
Euterreinigung trocken ☐ feucht ☐ garnicht ☐
Pro Kuh 1 Lappen ☐ ja ☐ nein
mit Einmaltüchern ☐ ja ☐ nein
Dippen/Sprühen ☐ ja ☐ nein
Dippmittel /Sprühmittel _____
Effektivität des Dippens/Sprühens
☐ 90-100%ig ☐ nicht alle 4 Zitzen ausreichend ☐ 50%ig

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Zwischendesinfektion ☐ immer ☐ sporadisch ☐ garnicht
☐ Peressigsäure ☐ Wasserdampf
☐ sonstige_____

Handschuhe ☐ ja ☐ nein
Ärmelschoner ☐ ja ☐ nein
Schürze ☐ ja ☐ nein

Routine Mastitiskühe

Melken als Gruppe am Ende ☐ ja ☐ nein
Desinfektion Melkzeug ☐ ja ☐ nein
Händedesinfektion ☐ ja ☐ nein

Vertränkung Hemmstoffmilch ☐ ja ☐ nein
Erhitzung der Tränkemilch ☐ ja ☐ nein

5. Kuhkomfort (Umwelt)

Stallgebäudetyp _____

Tier-Fressplatz Verhältnis _____

Tier-Liegeplatz Verhältnis _____

Liegeboxen

Einstreumaterial_____

Typ ☐Hoch- ☐Tief- ☐Hochtiefbox

6. Mastitis

Wann wird behandelt? _____

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Therapie
(Mittel, Therapiedauer, Applikationsart)
-
_____
_____
_____

Erregerbestimmung _____
(Leitkeime im Betrieb)

Wie wird mit S. aureus Mastitiden verfahren?

Selektion	☐ ja	☐ nein
Behandlung	☐ ja	☐ nein
Merzung	☐ ja	☐ nein

Wie wird mit MRSA Mastitiden verfahren?

Selektion	☐ ja	☐ nein
Behandlung	☐ ja	☐ nein
Merzung	☐ ja	☐ nein

Impfung (STARTVAC - HIPRA) ☐ ja ☐ nein

Imrestor (GCSF) ☐ ja ☐ nein

7. Tiergesundheit (Problembereiche)

Klauen/Gliedmaßen	☐ ja	☐ nein	(_____%)
Ketose	☐ ja	☐ nein	(_____%)
Milchfieber	☐ ja	☐ nein	(_____%)
Metritis	☐ ja	☐ nein	(_____%)
Endometritis	☐ ja	☐ nein	(_____%)
Stoffwechsel	☐ ja	☐ nein	(_____%)
Nachgeburtsverhalten	☐ ja	☐ nein	(_____%)
Sterilitäten	☐ ja	☐ nein	(_____%)

Sonstige _____

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8. Automatische Melksysteme (AMS)

Datum der Umstellung auf AMS _____

Stallneubau ☐ ja ☐ nein

Eutergesundheit vor der Umstellung ☐ schlechter ☐ genauso ☐ besser

Milchleistung vor der Umstellung ☐ schlechter ☐ genauso ☐ besser

Untersuchung von Milchproben vor Umstellung ☐ ja ☐ nein

Zukauf von Tieren für Umstellung ☐ ja ☐ nein Anzahl:_____

Untersuchung Milchproben? ☐ ja ☐ nein

Abgang von Tieren bei Umstellung ☐ ja ☐ nein Anzahl:_____

Kuhverkehr ☐ gelenkt ☐ frei sonstige_____

Hersteller ☐ Gea ☐ Delaval ☐ Lely ☐ Boumatic ☐ Lemmer Fullwood

☐ Dairymaster sonstige_____

Typ ☐ Monobox ☐ Karussell

technischer Support bei Störung ☐ <3h ☐ >3h ☐ unzuverlässig

Technische Schulung ☐ zufriedenstellend ☐ wenig hilfreich ☐ keine

Milchanalyse Funktionen ☐ Milchmenge- viertelindividuell ☐ ja ☐ nein
☐ Leitfähigkeit ☐ Farbe ☐ Blut ☐ Zellzahl
☐ Inhaltsstoffe ☐ Milchtemperatur

Milchmenge (l) je Roboter/Tag &Jahr _____

Melkende Kühe je Roboter _____

Melkungen/ Tag _____

Melkdauer/Kuh _____

Abbrüche (%) _____%

Bundesinstitut für Risikobewertung

Zitzenvorbereitung ☐ waschen ☐ bürsten ☐ keine

Hauptreinigungsgänge/Tag _____

Puffertank ☐ja ☐ nein

Zwischendesinfektion ☐ja ☐ nein
☐ Peressigsäure ☐ Wasserdampf
sonstige_____

Dippen/Sprühen ☐ ja ☐ nein
Dippmittel /Sprühmittel_____
Effektivität des Dippens/Sprühens
☐ 100%ig ☐ nicht alle 4 Zitzen ausreichend ☐ 50%ig

Backup Melkanlage ☐ja ☐ nein

Mastitis:
Erkennungsrate durch Roboter _____%
Wie wird mit Mastitis Kühen weiter verfahren?_____
_____
_____
_____
Wie wird mit behandelten Kühe weiter verfahren?_____
_____
_____
_____

Wann kommen Frischabkalber in Roboter?
_____

Wie wird das Erstkolostrum ermolken?
_____