J. Dairy Sci. 103:11806–11819
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 AMS	No (parlor) AMS (brush)	No AMS	No (AMS and parlor)	No	90–100	No
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. aureus* (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 gloves 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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