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

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.

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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 METHODS

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 internal 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

MRR-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.)	Test liners (no.)	Calf feeder (no.)	
<i>Staph. sciuri</i>	32	1	29	8	4	4	3	3	4	88
<i>Staph. lentus</i>	1		18	18	6	1	1	1	1	47
<i>Staph. fleuretii</i>	10			2	2	2		1		17
<i>Staph. epidermidis</i>	5	1	4	1		2		1		1
<i>Staph. haemolyticus</i>	6	2	2				1			11
<i>Staph. cohnii</i>	6							1		7
<i>Staph. capitis</i>										1
<i>Staph. vitulinus</i>				1	1					2
<i>Staph. kloosii</i>						1				1
<i>Staph. spp.</i> ²	17	4	6	5	1	1				33
Total	77/2,347	8/19	59/203	35/191	14/191	12/170	5/20	7/20	5/9	222/3,167
No. ³	3.3	42.1	29.1	18.3	7.3	7.1	25	35	55.6	7.0

¹MF = 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.

³MR-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

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

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

³Low = <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			
	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.)	Total (no.)
<i>Staph. cohnii</i>	120	2		4	2	6	2	2		138
<i>Staph. pettenkoferi</i>	1			1	1					3
<i>Staph. xylosus</i>	2									2
<i>Staph. saprophyticus</i>	1									1
<i>Staph. spp.</i> ²	21		1	1	1			2		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

¹MF = 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.

³MR-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 extent.

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 (Mahmod 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 SCCmec 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 SCCmec type (V) as the MRSA on these farms. On one more farm, SCCmec type V was detected in MR *Staph. cohnii*, MRSE, and MRSA. It might be hypothesized that SCCmec 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 SCCmec elements and thus give hints for transmission events, in-depth analyses of the SCCmec DNA sequences should be performed. Because the majority of SCCmec 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) were 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*.

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