Occurrence and Characteristics of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* in Quarter Milk Samples From Dairy Cows in Germany

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The aim of this study was to identify and characterize LA-MRSA in a very recent collection of staphylococci isolated from bovine quarter milk samples. All milk samples \((n = 14,924)\) sent to the MBFG in March 2017 were included into this study. The samples originated from 3,887 cows with 3,367 samples from 2,280 animals being positive for bacteria, prototheca and/or yeast. The second most common infectious agent was *Staphylococcus* and 659 isolates were investigated. *Staphylococcus aureus* was confirmed by PCR for the *spa* gene. A CC398-specific PCR was performed for all *S. aureus* isolates. Susceptibility to penicillin was tested for all isolates by agar disk diffusion. All oxacillin resistant isolates were analyzed by microarray and tested for their susceptibility to 30 antimicrobial agents. Of the isolates 372 were *S. aureus* from Germany with 214 isolates being not epidemiologically related. Among the independent isolates nine were identified as oxacillin resistant. In addition five isolates epidemiologically related to these nine were MRSA. Among the remaining isolates seven independent isolates belonged to CC398. In conclusion this study showed a high detection rate of staphylococci in bovine quarter milk samples. In contrast MRSA was rarely detected and belonged in all cases to CC398. Only 7/214 MSSA (3.3%) belonged to this CC.

**Keywords:** staphylococci, antimicrobial resistance, mecA, oxacillin resistance, mastitis, Germany, dairy, animal
INTRODUCTION

Staphylococci belong to the most important bacterial causes of mastitis in dairy cattle worldwide. Among the isolates identified during routine diagnostics on milk samples in Germany the resistance situation is still favorable, but the number of resistant staphylococci seems to be increasing (GERM-Vet, 2016). The same situation is seen worldwide as documented for example during routine diagnostics on milk samples in Germany the same situation is seen worldwide as documented for example during routine diagnostics on milk samples in Germany the mastitis in dairy cattle worldwide. Among the isolates identified Staphylococci belong to the most important bacterial causes of LA-MRSA in German Dairy Cows. Kadlec et al. (2018). The remaining 307 isolates originated from 43 farms which sent in four or more milk samples being positive for staphylococci. The samples from the dairy farms were sent in partly one order and partly on different days. The farm which contributed the highest number of Staphylococcus isolates had sent in samples on 8 days and 31 staphylococci were detected in the samples from 21 different animals.

Within the framework of the study Staphylococcus isolates were confirmed as S. aureus by a PCR for the spa gene. The PCR used for this approach was the one used for spa typing (Kadlec et al., 2009). In isolates negative in this PCR a part of the 16s rDNA was amplified by another PCR, the product sequenced and compared via blastN (Weiß et al., 2013). Susceptibility to penicillin was tested by agar disk diffusion with 10U penicillin G disks and susceptibility to oxacillin by broth dilution according to the Clinical and Laboratory Standards Institute (CLSI, 2018). The S. aureus strains ATCC 25923 as well as ATCC 29213 were used as quality control, respectively (CLSI, 2018). Moreover, MICs to ciprofloxacin (16–0.008 mg/L), clindamycin (64–0.03 mg/L), enrofloxacin (16–0.008 mg/L), erythromycin (32–0.015 mg/L), gentamicin (256–0.12 mg/L), kanamycin (128–0.03 mg/L), linezolid (64–0.03 mg/L), oxacillin (8–0.0015 mg/L), pirlimycin (64–0.03 mg/L), quinupristin/dalfopristin (32–0.015 mg/L), tetracycline (256–0.12 mg/L), trimethoprim/sulfamethoxazole (SXT) (32/608–0.015/0.3 mg/L), trimethoprim (256–0.12 mg/L), and sulfamethoxazole (1024–1 mg/L) were determined by broth dilution for the oxacillin-resistant isolates. The oxacillin-resistant isolates were characterized by the staphype type microarray (Monecke et al., 2011). Presence of antimicrobial resistance genes was tested by previously described PCR assays or by the staphype type microarray (Feßler et al., 2010).

MATERIALS AND METHODS

In this study all milk samples (% = 14,924) send to the MBFG in March 2017 were included. MBFG is among the diagnostic laboratories in Germany with the highest number of milk samples analyzed. MBFG is located in the northern part of Germany, but receives samples from all over the country as well as from neighboring countries. The quarter milk samples were sent for bacteriological analysis. In most of the cases a milk sample is sent from each lactating quarter, thus four samples per cow. In the majority of cases at least one of the quarters showed a clinical mastitis. Other reasons for sending milk samples are subclinical mastitis, control after mastitis treatment or a control prior to drying off. The laboratory procedure includes direct plating on sheep blood agar without any enrichment or selective steps. The milk samples originated from 3,867 cows with 3,367 samples from 2,280 animals being positive for bacteria, prototheca and/or yeast. Second most common infectious agent was Staphylococcus and 659 isolates were investigated. The isolates originated from 291 dairy farms (Table 1). While 166 Staphylococcus isolates were from farms which contributed only one isolate each, 60 isolates came from 30 other farms which accounted for two Staphylococcus isolates each and 22 farms contributed three Staphylococcus isolates. The remaining 307 isolates originated from 43 farms which sent in four or more milk samples being positive for staphylococci. The samples from the dairy farms were sent in partly one order and partly on different days. The farm which contributed the highest number of Staphylococcus isolates had sent in samples on 8 days and 31 staphylococci were detected in the samples from 21 different animals.

RESULTS

Among the 659 Staphylococcus isolates, 372 were S. aureus. In some cases S. aureus was isolated from more than one quarter milk sample of the respective cow. In some other cases S. aureus was isolated from more than one animal in the same herd. Such isolates were also not considered as epidemiologically independent. Finally, this collection comprised 214 epidemiologically independent S. aureus isolates. In addition, in ten cases in which S. aureus isolates were considered as epidemiologically related to each other, the isolates showed differences in their characteristics. For example we had cases, in which in one quarter sample from a cow MRSA was found and in another sample from the same animal MSSA was identified (Table 2). Susceptibility testing revealed 9/214 oxacillin resistant epidemiologically independent isolates. In addition, five of the epidemiologically related isolates were resistant to oxacillin. In three cases the same animal had a second indistinguishable MRSA in a different quarter sample. In one case the same...
MRSA was identified in a sample from a different cow. Two indistinguishable MRSA isolates were seen in two animals from the same farm in the fifth case (isolate Wu490), resulting in a total of 10 different MRSA isolates (Table 2). All ten isolates were tetracycline resistant with slight differences in tetracycline resistance genes: Two and one isolates lacked tet(K) or tet(M), respectively, whereas two isolates carried tet(L). Four MRSA were macrolide and lincosamide resistant encoded by erm(A). All four had a very high spectinomycin MIC and harbored the resistance gene spc. Three of these four isolates were also trimethoprim and sulfonamide-resistant. In addition to mecA, all MRSA isolates carried blaZ (Table 2).

All MRSA were positive in the PCR for CC398. The microarray underlined this finding and classified them also as MRSA CC398. All MRSA isolates had the methicillin resistance gene mecA on a SCCmec element type V. All isolates had capsule type 5 and agrI. Enterotoxin genes were not detected in the LA-MRSA isolates. The ten MRSA isolates varied only in a few of the genes, which are included in the microarray (Table 2).

Among the ten MRSA CC398, one isolate originated from a farm in which no other staphylococci were detected. The remaining MRSA CC398 were from farms in which staphylococci isolates were isolated from two to nine cows. In one case (isolate ID: Wu69-1) the MRSA was isolated from the same quarter as a Staphylococcus succinicus, which was methicillin-susceptible.

In addition to LA-MRSA, seven of 214 (3.3%) independent isolates and 11/358 (3.1%) isolates in total among the methicillin-susceptible S. aureus isolates belonged to CC398 according to the PCR. Three of these were from farms with LA-MRSA (Table 2). In two cases the LA-MSSA isolate was identified from the same cow, but from a different quarter than the LA-MRSA, in the remaining case two indistinguishable LA-MSSA were isolated from different quarters from a cow without LA-MRSA.

### DISCUSSION

In total 214 independent S. aureus isolates of the 372 isolates form Germany were studied in detail. It seems to be common knowledge, that S. aureus in dairy cattle is a herd health problem and that S. aureus spreads within the farm. Insights into single farms underline this assumption (Feßler et al., 2012). From a number of dairy farms more than one S. aureus was isolated. In general all isolates from the same farm were not considered as independent. For the counted numbers we considered such isolates as being epidemiologically related. However, we saw even single animals carrying two different S. aureus. Based on the epidemiological relatedness, only selected isolates were tested for their susceptibility. Therefore it cannot be excluded, that more isolates would show differences to isolates presumed to be related. Among all isolates tested for their susceptibility, ten isolates showed different susceptibility profiles compared to isolates considered as epidemiologically related. These ten isolates were also included into further tests. Moreover, our decision was based solely on susceptibility profiles, further in-depth analysis could also reveal differences between S. aureus isolates from the same animal or the same farm (Kadlec et al., 2015). In general this is a limit of most of the studies performed. It is definitely a waste of resources to investigate copy isolates, but it is unknown how high the number of animals is, that carry a mix of bacteria belonging to the same species. During routine diagnostics from a culture consisting of a single species a single colony is picked and used for further analysis. In our study, however, we saw isolates with different characteristics from the same quarter, the same animal or the same farm. This study confirms, the presence of different S. aureus isolates on one farm and the presence of different S. aureus isolates in different quarters of the udder of the same animal. It suggests the possibility of the presence of different S. aureus in the same sample in very rare cases.

The identification of 9/214 independent isolates as oxacillin-resistant clones fits well into the data form the German National Resistance monitoring program GERM-Vet, investigating a representative German collection. In GERM-Vet nine of 205 S. aureus isolates collected in 2013/2014 were identified and confirmed as MRSA (GERM-Vet, 2016). It is also in accordance with the identification of 19/430 isolates from Belgium (Bardiaux et al., 2013). However, in the latter study one of the MRSA isolates did not belong to CC398. As in this study only CC398 MRSA were identified among isolates from Switzerland (Sakwinska et al., 2011). Whereas MRSA from bovine mastitis most commonly belong to CC398, MSSA from cows belong to other CCs (Holmes and Zadoks, 2011). This was also seen in the present study with only 3% of the MSSA belonging to CC398. Although the number of MRSA CC398 was low in this study, the finding of such isolates is still not so common and worthwhile to mention. In general the rate of MRSA among isolates from bovine mastitis is still low, but seems to be increasing and should be monitored.

The resistance phenotypes and genotypes resembled those previously seen in LA-MRSA (Kadlec et al., 2012). However, this study also underlines that in isolates from mastitis you still find a favorable resistance situation. This is obvious by two results of
### TABLE 2 | Characteristics of the 10 LA-MRSA isolates (CC398, SCCmec type V) from dairy cows from Germany.

| Isolate ID | Date       | Other cows with staphylococci from the same farm | Resistance phenotype | Resistance genotype | Differences in the microarray<sup>b</sup> |
|------------|------------|------------------------------------------------|----------------------|--------------------|------------------------------------------|
|            |            |                                                 |                      | agrB agrC ssl01/set6 ssi05/set3 sdrC mprF |
| Wu65       | 2017-03-03 | 2 cows 1: MRSA \(n = 2\), Wu65 2: MRSA         | BLA, TET             | mecA, blaZ, tet(K), tet(M) | + + + + + + + |
| Wu69-1     | 2017-03-03 | 2 cows 1: MRSA (Wu69-1), S. succinus (same quarter) 2: MSSA \(n = 2\) | BLA, TET             | mecA, blaZ, tet(K), tet(M) | + + + - + + |
| Wu116      | 2017-03-04 | 2 cows 1: MRSA \(n = 2\), Wu116, S. simulans 2: MSSA | BLA, TET             | mecA, blaZ, tet(K), tet(M) | + + + - + + |
| Wu250      | 2017-03-03 | 3 cows 1: MRSA (Wu250) 2: S. chromogenes 2: MSSA (other cow, 2017-03-16) | BLA, TET             | mecA, blaZ, tet(K), tet(M) | + - + - - - |
| Wu306      | 2017-03-15 | 3 cows 1: MRSA (Wu306) 2: MSSA 3: S. chromogenes | BLA, TET, ML, SPE    | mecA, blaZ, tet(K), tet(M), emr(A), spc | + + + - + - |
| Wu488      | 2017-03-24 | 7 cows 1: S. chromogenes 2: MRSA (Wu488), MSSA (CC398) 3: S. chromogenes 4: MRSA \(n = 2\), Wu490, MSSA (CC398) 5: S. simulans 6: MSSA 7: MSSA | BLA, TET, ML, TMP, SUL, SPE | mecA, blaZ, tet(M), tet(L), emr(A), dfrK, spc | + + + - + + |
| Wu490      | 2017-03-24 | cf. Wu488                                        | BLA, TET, ML, TMP, SUL, SPE | mecA, blaZ, tet(K), tet(L), emr(A), dfrK, spc | + - + - - ? - |
| Wu609      | 2017-03-29 | 9 cows 1: MSSA \(n = 3\) 2: MSSA \(n = 2\) 3: MRSA Wu609 4: MSSA 5: MSSA, S. haemolyticus 6: S. rostrii 7: MSSA \(n = 2\) 8: MSSA \(n = 2\) 9: MSSA \(n = 3\) | BLA, TET             | mecA, tet(K), tet(M) | + + + - + + |
| Wu640      | 2017-03-30 | 5 cows 1: MSSA 2: MSSA 3: MSSA \(n = 2\), CC398 4: MRSA Wu640, MSSA 5: MSSA \(n = 3\) | BLA, TET             | mecA, blaZ, tet(K), tet(M), emr(A), spc | + + + - + - |
| Wu658      | 2017-03-31 | 1 cow: MRSA Wu658                               | BLA, TET             | mecA, blaZ, tet(M) | - - - - - - |

<sup>a</sup>BLA, β-lactams; TET, tetracycline; ML, macrolides/lincomamides; TMP, trimethoprim; SUL, sulfonamides; SPE, spectinomycin. <sup>b</sup>? an ambiguous result was seen in the microarray, it is unclear whether the isolate harbors this gene or not.
the study: (1) The number of MRSA was low. (2) Six of the ten different LA-MRSA isolates were resistant only to β-lactams and to tetracyclines. In contrast, the presence of multiresistant LA-MRSA was also seen with the four remaining isolates showing resistance to several classes of agents. The identification of multi-resistant strains among isolates from milk samples is alarming and requires strict monitoring schemes.

Among the LA-MRSA isolates of this study no isolate harbored an enterotoxin gene. LA-MRSA isolates have been shown to harbor very rarely enterotoxin genes (Kadlec et al., 2009; Argudín et al., 2013). Thus, the result of this study fits well to the known situation. Differences in genes associated to virulence are in general alarming. The acquisition of virulence genes is a worrying possibility for this type of MRSA. LA-MRSA can adapt to different hosts and seem to be very flexible in the acquisition of resistance genes (Kadlec et al., 2012). In case they make use of their genomic flexibility and virulence properties start to increase, such isolates would have another big advantage. In this study still a favorable situation is seen with only very few differences in global regulator genes and in genes associated to virulence.

REFERENCES
Argudín, M. A., Cariou, N., Salandre, O., Le Guennec, J., Nemeighaer, S., and Butaye, P. (2013). Genotyping and antimicrobial resistance of Staphylococcus aureus isolates from diseased turkeys. Avian Pathol. 42, 572–580. doi: 10.1080/03079457.2013.854308
Bardiau, M., Yamazaki, K., Duprez, J. N., Taminiau, B., Mainil, J. G., and Ote, K. (2011). Characterization of antimicrobial resistance and the characterization of antimicrobial resistance and the characterization of methicillin-resistant Staphylococcus aureus (MRSA) isolated from milk of bovine mastitis. Lett. Appl. Microbiol. 57, 181–186. doi: 10.1111/lam.12099

CLSI (2018). Clinical and Laboratory Standards Institute. (2018). Clinical and Laboratory Standards Institute - Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals, CLSI Supplement VET08, 4th Edn. Wayne, PA: CLSI.

Cuny, C., Köck, R., and Witte, W. (2013). Livestock associated MRSA (LA-MRSA) and its relevance for humans in Germany. Int. J. Med. Microbiol. 303, 331–337. doi: 10.1016/j.ijmm.2013.02.010

de Jong, A., Garch, F. E., Simjee, S., Moyaert, H., Rose, M., Youala, M., et al. (2018). Monitoring of antimicrobial susceptibility of udder pathogens recovered from cases of clinical mastitis in dairy cows across Europe: vetpath results. Vet. Microbiol. 213, 73–81. doi: 10.1016/j.vetmic.2017.11.021

Felffer, A. T., Olde Riekerink, R. G., Kadlec, K., and Schwarz, S. (2016). Genomic investigation of methicillin-resistant Staphylococcus aureus ST398 from diseased turkeys. J. Antimicrob. Chemother. 71, 619–625. doi: 10.1093/jac/dkw576

Felffer, A. T., Kadlec, K., Ehrlich, R., Monecke, S., Steinacker, U., Kaspar, H., Mankertz, J., et al. (2009). Diversity of antimicrobial resistance pheno- and genotypes of methicillin-resistant Staphylococcus aureus ST398 from diseased swine. J. Antimicrob. Chemother. 64, 1156–1164. doi: 10.1093/jac/dkq021

Kadlec, K., Felffer, A. T., Hauschild, T., and Schwarz, S. (2012). Novel and uncommon antimicrobial resistance genes in livestock-associated methicillin-resistant Staphylococcus aureus. Clin. Microbiol. Infect. 18, 745–755. doi: 10.1111/j.1469-0691.2012.03842.x

Kadlec, K., Wendlandt, S., Felffer, A. T., and Schwarz, S. (2015). “Methods for the detection of antimicrobial resistance and the characterization of Staphylococcus aureus isolates from food producing animals and food of animal origin,” in Antimicrobial Resistance and Food Safety Methods and Techniques, eds C.- Y. Chen, X. Yan, and C. R. Jackson (Cambridge: Academic Press, Elsevier), 207–226.

Klibi, A., Maaroufia, A., Torres, C., and Jouini, A. (2018). Detection and characterization of methicillin-resistant and susceptible coagulase-negative staphylococci in milk from cows with clinical mastitis in Tunisia. Int. J. Antimicrob. Agents 52, 930–935. doi: 10.1016/j.ijantimicag.2018.07.026

Liu, H., Li, S., Meng, L., Dong, L., Zhao, S., Lan, X., et al. (2017). Prevalence, antimicrobial susceptibility, and molecular characterization of Staphylococcus aureus isolated from dairy herds in northern China. J. Dairy Sci. 100, 8796–8803. doi: 10.3168/jds.2017–13370

Monecke, S., Coombs, G., Shore, A. C., Coleman, D. C., Akpaka, P., Borg, M., et al. (2011). A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant Staphylococcus aureus. PLoS One 6:e17936. doi: 10.1371/journal.pone.0017936

Nemeighaer, S., Argudin, M. A., Haesebrouck, F., and Butaye, P. (2014). Epidemiology and molecular characterization of methicillin-resistant Staphylococcus aureus nasal carriage isolates from bovines. BMC Vet. Res. 10:153. doi: 10.1186/1746-6148-10-153

Ronco, T., Klaas, I. C., Stegger, M., Svenness, L., Astrup, L. B., Farre, M., et al. (2018). Genomic investigation of Staphylococcus aureus isolates from bulk tank milk and dairy cows with clinical mastitis. Vet. Microbiol. 215, 35–42. doi: 10.1016/j.vetmic.2018.01.003

Sakwinska, O., Moreillon, M., Morisset, D., Waldvogel, A., and Moreillon, P. (2011). Staphylococcus aureus host range and human-bovine host shift. Appl. Environ. Microbiol. 77, 5908–5915. doi: 10.1128/AEM.00238-11

CONCLUSION
This study showed a high detection rate of staphylococci in bovine quarter milk samples. Among the staphylococcal isolates, LA-MRSA was rarely detected. All 14 LA-MRSA belonged to CC398, whereas only 11/358 MSSA (3.1%) belonged to this clonal complex.

AUTHOR CONTRIBUTIONS
All authors participated in the study and provided additions and corrections to the manuscript. KK wrote the first draft.

ACKNOWLEDGMENTS
The authors thank Vivian Hensel, Ute Beermann, Regina Ronge, Christian Moelders, Alina Kirchhoff, and Henrik Senge for excellent technical assistance.
Vanderhaeghen, W., Cerpentier, T., Adriaensen, C., Vicca, J., Hermans, K., and Butaye, P. (2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. Vet. Microbiol. 144, 166–171. doi: 10.1016/j.vetmic.2009.12.044

Weiß, S., Kadlec, K., Feßler, A. T., and Schwarz, S. (2013). Identification and characterization of methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus* and *Staphylococcus pettenkoferi* from a small animal clinic. Vet. Microbiol. 167, 680–685. doi: 10.1016/j.vetmic.2013.07.036

Wendlandt, S., Feßler, A. T., Monecke, S., Ehrlich, R., Schwarz, S., and Kadlec, K. (2013). The diversity of antimicrobial resistance genes among *Staphylococci* of animal origin. Int. J. Med. Microbiol. 303, 338–349. doi: 10.1016/j.ijmm.2013.02.006

**Conflict of Interest Statement:** ME and TP were employed by the Dairy Herd Consulting and Research Company (MBFG).

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest, however KK is meanwhile employed by the MBFG.

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