Comparison of phenotypic and genotypic antimicrobial resistance patterns associated with *Staphylococcus aureus* mastitis in German and Danish dairy cows

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ABSTRACT

Staphylococcus aureus is one of the most common pathogens associated with bovine mastitis in Germany and Denmark. Successful therapy is strongly linked to the susceptibility of the pathogen to the administered antimicrobial. An increase in resistant pathogens in human and veterinary medicine has become a concern worldwide and hampers therapy due to reduced susceptibility. In the present study, susceptibility testing was performed for 85 and 93 *S. aureus* isolates originating from mastitis cases on 12 German and 8 Danish dairy farms, respectively. Phenotypic examination was performed by detection of minimal inhibitory concentration (MIC) values using the broth microdilution method, followed by genotypic investigations of the *blaZ* and *mecA* resistance genes via PCR. The tested antimicrobials were the most frequently used β -lactams in German and Danish dairy farms, including cefquinome, cefoperazone, cephapirin, penicillin, oxacillin, cloxacillin, amoxicillin-clavulanic acid, and cephalixin-kanamycin. Special attention was paid to varying therapy concepts because, in Germany, third- and fourth-generation cephalosporins have been predominantly used in mastitis therapy, whereas in Denmark, restrictive use of penicillin is followed by a general avoidance of cephalosporins. Differences in MIC values were mainly based on determined MIC₉₀ values (MIC at which 90% of isolates are inhibited). In general, Danish *S. aureus* isolates were inhibited at comparatively lower MIC₉₀ values than *S. aureus* isolated from German dairy

farms for most β -lactams. No differences were observed regarding cefquinome, because both German and Danish isolates exhibited MIC₅₀ and MIC₉₀ values of 0.5 and 1 μ g/mL, respectively. In contrast, the MIC₉₀ for penicillin against German and Danish *S. aureus* were 0.5 and ≤ 0.06 μ g/mL, respectively. Resistance genes (*blaZ*, *mecA*) were only detected in German *S. aureus* isolates on 3 dairy farms in Germany. A total of 5 isolates tested positive for both *blaZ* and *mecA*, whereas 1 isolate carried the *blaZ* resistance gene only. A direct correlation between frequently used antimicrobials and reduced susceptibility could not be determined based on results of the present study. In addition to further research to determine factors associated with resistance development, we emphasize the urgent need for internationally standardized clinical breakpoints to assess resistance situations more accurately.

Key words: *Staphylococcus aureus*, mastitis, minimum inhibitory concentration, *blaZ*, *mecA*

INTRODUCTION

In dairy farming, *Staphylococcus aureus* plays an important role as a major pathogen of clinical and sub-clinical mastitis. Bovine mastitis is generally treated with β -lactams, including the penicillin and cephalosporin groups (Tenhagen et al., 2006; Pol and Ruegg, 2007; Ziesch et al., 2018). Resistance to these β -lactams is based on 2 different resistance mechanisms. One is encoded by the *blaZ* resistance gene and acts through enzymatic inactivation by β -lactamases. These β -lactamases are capable of hydrolysing the β -lactam, resulting in inactive metabolites. The other mechanism is encoded by the *mecA* gene, which induces the formation of an altered penicillin binding protein (PBP) to which antimicrobials have a greatly reduced affinity and thus lose their efficacy (Schwarz and Chaslus-Dancla,

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2001; Wendlandt et al., 2013). Mutual transmission of methicillin-resistant *S. aureus* (MRSA) between dairy cows and humans, especially those in close contact with animals, is suspected (Feßler et al., 2012, Lim et al., 2013, Unnerstad et al., 2018). In this context, the European Medicines Agency (EMA) classified antimicrobials according to their risk of resistance development and special relevance for human health. Thus, use of third- and fourth-generation cephalosporins (category 2) in veterinary medicine is considered to pose a higher risk to human health than natural and narrow-spectrum penicillins (category 1; EMA, 2014). In Germany, the total amount of antimicrobial agents dispensed in veterinary medicine decreased by 972.6 t (57%) between 2011 and 2017. Although the whole group of penicillins decreased by 258.9 t, cephalosporins of all generations remained relatively constant (Wallmann et al., 2018). According to data on antimicrobial intramammary tubes sold in the first quarter of 2017, cefoperazone and cefquinome accounted for more than one-third of all antimicrobials relevant in mastitis therapy (unpublished data provided by the Association for Consumer Research, GfK, Nürnberg, Germany). Since March 2018, susceptibility testing has been required prior to treatment with third- and fourth-generation cephalosporins (BMJV, 2018).

In contrast, Nordic countries such as Denmark, Norway, and Sweden pursue restrictive antimicrobial use by avoiding cephalosporins as far as possible. Thus, treatment of *S. aureus* with β -lactamase-resistant antimicrobials is only indicated “in cases of severe animal welfare conditions” induced by β -lactamase-positive bacteria. Otherwise, only supportive therapy or treatment with penicillin, in case of β -lactamase-negative pathogens, is the first choice of mastitis treatment according to the jointly created “Nordic Guidelines for Mastitis Therapy” (Landin et al., 2011). Moreover, the Danish government established thresholds for antimicrobial consumption, and blanket dry-cow therapy is prohibited without prior bacterial diagnosis (Carmo et al., 2017). Denmark thus reduced its overall antimicrobial intake by more than 16 t between 2013 and 2017 and has steadily moved away from the use of cephalosporins. Intramammary antimicrobial therapy is performed predominantly by use of β -lactamase-sensitive penicillins, which represented 59.8% of all antimicrobials used for intramammary treatment in 2017. The β -lactamase-stable penicillins such as oxacillin and extended-spectrum penicillins (including combinations with β -lactamase inhibitors) made up 20.3% and cephalosporins 10.5% of the total. Regarding the cephalosporins used for intramammary application, the ratio of administered first-generation cephalosporins to those of third- and fourth-generation was 96.6% to 3.4% (DANMAP, 2018).

Because of the clear contrast in antimicrobial use for mastitis therapy in Germany and Denmark, the objective of this study was to identify whether this contrast was reflected in different susceptibility patterns of *S. aureus* and considering the presence of *blaZ* and *mecA* resistance genes.

MATERIALS AND METHODS

Collection of Milk Samples and Microbiological Identification of Isolates

A total of 178 *S. aureus* isolates from IMI of dairy cows were used for susceptibility testing and genotypic investigations. The German and Danish farms were representative of milk-producing dairy farms in the respective countries, with predominant use of third- and fourth-generation cephalosporins (Germany) or penicillin (Denmark). The 85 German *S. aureus* isolates were randomly selected from the strain collection of the University of Applied Sciences and Arts (Hannover, Germany) and originated from quarter foremilk samples that had been sent to the microbiological laboratory for routine diagnosis. Milk samples were collected between February 2016 and January 2018 on 12 dairy farms located in northern Germany. The herd size of participating dairy farms ranged from 85 to 1,325 cows and the Holstein Friesian breed predominated on these dairy farms. Annual milk production ranged from 8,726 to 12,353 kg of ECM per cow and bulk tank SCC varied from 87,000 to 328,000 cells/mL.

Milk samples from infected quarters of cows suffering from clinical mastitis were taken aseptically in accordance with the guidelines of the German Veterinary Medical Association (2009) by using sterile plastic tubes. Immediately after sampling, tubes were cooled to below 6°C and sent to the microbiological laboratory of the University of Applied Sciences and Arts for etiological diagnosis. Analyses of samples including culture and identification of pathogens were conducted in accordance with the methods described by the German Veterinary Medical Association (2009). A quantity of 10 μ L of each milk sample was plated on a quadrant of esculin blood agar (Oxoid Deutschland GmbH, Wesel, Germany) and incubated at 37°C under aerobic conditions. After 24 and 48 h of incubation, colonies were first investigated concerning their morphology and hemolysis patterns. In addition to Gram staining, examination of cell morphology, and catalase activities (3% H₂O₂; Merck AG, Darmstadt, Germany), the clumping factor test (DiaMondiaL Staph Plus Kit, Virotech Diagnostics GmbH, Rüsselsheim am Main, Germany) is useful for initial differentiation of presumptive *S. aureus* isolates. The *S. aureus*-specific *nuc* gene was detected in accor-

dance with Saiful et al. (2006) to confirm the presence of *S. aureus* in milk samples of IMI (Saiful et al., 2006).

A total of 93 *S. aureus* isolates originating from milk samples of dairy cows with subclinical mastitis ($\geq 200,000$ cells/mL) in Danish dairy herds were provided by the University of Copenhagen (Denmark). Danish *S. aureus* isolates were obtained within the scope of previous research projects investigating contagious mastitis pathogens in Danish dairy herds (Mahmmod et al., 2018; Svennesen et al., 2019). For this purpose, *S. aureus* isolates originating from 8 dairy farms were isolated from February 2017 to April 2017 by project researchers of the University of Copenhagen (Denmark). Between 30 and 40 dairy cows with an elevated SCC ($\geq 200,000$ cells/mL) but lacking clinical symptoms were randomly sampled from each dairy herd. At sampling collection, herd sizes ranged from 198 to 344 dairy cows with an estimated milk production between 9,024 and 11,909 kg of ECM per cow and a bulk tank SCC ranging from 183,000 to 338,000 cells/mL. Danish Holstein was the predominant breed on all dairy farms; 2 herds consisted entirely of Danish Holstein animals. Sample collection and identification of *S. aureus*, including bacterial culturing and MALDI-TOF assay, were performed and previously described by the project researchers (Mahmmod et al., 2018; Svennesen et al., 2019). Of the total number of harvested *S. aureus* isolates from the projects, 93 were randomly selected for inclusion in the current study. After identifying all pathogens as *S. aureus*, German and Danish isolates were added to the strain collection of the University of Applied Sciences and Arts (Hannover, Germany) and stored at -80°C , with the addition of 20% glycerin, until susceptibility testing.

Antimicrobial Susceptibility Testing

The MIC is a measurement of a pathogen's susceptibility to an antimicrobial agent; it is defined as the lowest concentration at which bacterial proliferation is no longer visually apparent. Antimicrobial susceptibility testing of 178 *S. aureus* isolates was performed at the University of Applied Sciences and Arts in Hannover (Germany). The tested antimicrobial agents were selected based on the substances most frequently used for mastitis therapy in Germany and Denmark, including the following β -lactams: cefquinome, cefoperazone, cephapirin, penicillin, oxacillin, cloxacillin, amoxicillin-clavulanic acid (4:1), and cephalixin-kanamycin (2:1). The proportions given for the tested combinations (amoxicillin-clavulanic acid, cephalixin-kanamycin) refer to the ratio contained in intramammary tubes approved for mastitis treatment. Examination of MIC values was performed using the broth microdilution

method in accordance with the DIN 58940/ISO20776-1:2006 protocol (DIN, 2007). Polystyrene sterile microtiter plates (Greiner Bio One GmbH, Frickenhausen, Germany) were used that contained a 2-fold dilution series of each antimicrobial substance ranging from 0.06 to 32 $\mu\text{g/mL}$.

Respective concentration levels were prepared using Mueller-Hinton broth (Carl Roth GmbH, Karlsruhe, Germany) as a liquid nutrient medium. Wells of the microtiter plates contained 100 μL of an antimicrobial solution to which 5 μL of an immediately prepared bacterial inoculum suspension was added. Positive controls (wells only containing Mueller-Hinton broth) as well as negative controls (wells without bacterial inoculation) were included in the microtiter plates, and *S. aureus* ATCC 29213 (Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was used as a reference strain. After incubating the microtiter plates for 16 to 20 h at 37°C , the MIC were determined as wells containing no visible turbidity, corresponding to the lowest concentration that inhibited bacterial growth. Here, MIC₅₀ and MIC₉₀ were defined as the lowest concentrations where growth of at least 50% and 90% of the tested bacteria are inhibited, respectively (DIN, 2007).

Determination of Presence of *blaZ* and *mecA* Resistance Genes

Extraction of bacterial DNA was performed by using the DNeasy Blood and Tissue Kit (Qiagen N.V., Venlo, the Netherlands). The PCR was carried out in a 25- μL reaction mix including 12.5 μL of ReadyMix Taq PCR Reaction Mix (Sigma-Aldrich GmbH, Steinheim, Germany), 20 pmol of primer, 5 μL of template, and H₂O for the no-template control. Amplification reactions were performed in a Stratagene Mx3005P qPCR System Thermocycler (Agilent Technologies Inc., Santa Clara, CA). Primers used to detect resistance genes as well as temperature profiles were programmed as previously described for *blaZ* (Vesterholm-Nielsen et al., 1999) and *mecA* (Saiful et al., 2006). The PCR products were directly stained with Midori Green Direct (Nippon Genetics Europe GmbH, Dueren, Germany) and separated in a 2% agarose gel at 100 V for 2 h. Results, including the distribution of isolates, MIC₅₀, MIC₉₀ values, and the presence of resistance genes, were calculated using descriptive statistics.

RESULTS

Results of MIC examinations for *S. aureus* isolates obtained from IMI of dairy cows are presented in detail in Table 1 and Table 2 for 85 German and 93 Danish *S.*

Table 1. Distribution of MIC of *Staphylococcus aureus* (n = 85) isolated from 12 German dairy farms¹

Antimicrobial ²	MIC (µg/mL)										
	≤0.06	0.125	0.25	0.5	1	2	4	8	16	32	>32
Cefquinome	—	—	4	<i>40</i>	35	5	1	—	—	—	—
Cefoperazone	—	—	—	19	12	<i>33</i>	15	2	4	—	—
Cephapirin	22	<i>31</i>	21	6	1	3	1	—	—	—	—
Penicillin	<i>64</i>	11	1	3	—	—	2	—	3	1	—
Cloxacillin	1	15	<i>30</i>	27	8	4	—	—	—	—	—
Oxacillin	—	13	<i>33</i>	22	12	1	1	3	—	—	—
AMC	5	31	<i>27</i>	11	5	6	—	—	—	—	—
CFX-K	—	—	—	—	2	<i>56</i>	27	—	—	—	—

¹MIC₅₀ and MIC₉₀ are shown in italics and bold, respectively.

²AMC = amoxicillin-clavulanic acid; CFX-K = cephalexin-kanamycin.

aureus isolates, respectively. In addition, the distribution of isolates according to determined MIC, MIC₅₀, and MIC₉₀ values are given (Tables 1 and 2). A direct comparison of MIC₅₀ and MIC₉₀ values of German and Danish *S. aureus* isolates is presented in Table 3.

Staphylococcus aureus Obtained from German Dairy Herds

Based on the determined MIC values, the distribution of the 85 German *S. aureus* isolates was wide for most β-lactams and extended over at least 5 dilution levels with the exception of cephalexin-kanamycin, ranging from 1 to 4 µg/mL. The widest distribution pattern was determined for penicillin, from ≤0.06 mg/mL to 32 mg/mL, with no inhibition of isolates at concentration of 1, 2, and 8 µg/mL. The highest MIC₅₀ and MIC₉₀ values were 2 and 4 µg/mL, respectively for cefoperazone and for the cephalexin-kanamycin combination. The MIC₉₀ values of cefquinome, cefoperazone, and cephalexin-kanamycin were 1 dilution higher than their MIC₅₀ values, whereas the MIC₉₀ of cephapirin, cloxacillin, oxacillin, and amoxicillin-clavulanic acid were 2 dilutions higher than their MIC₅₀ values. The lowest MIC₅₀ value was determined for penicillin, whereas

MIC₉₀ values (0.5 µg/mL) were lowest for penicillin as well as cephapirin. Because the MIC₅₀ value of penicillin was ≤0.06 µg/mL, the penicillin MIC₉₀ value was at least 3 dilutions higher (Table 1).

By PCR analysis, 5 isolates (5.9%) were positive for *mecA* and *blaZ*. Of these, 4 isolates originated from the same dairy farm (farm A), and 1 was isolated from another dairy farm in northern Germany (farm B). Besides these 5 isolates, 1 isolate originating from a different farm (farm C) was positive for *blaZ* only (Table 4). The distribution patterns of isolates originating from farms A and B extended over several dilution levels of the tested β-lactams. These were based on isolates that carried resistance genes and were inhibited at higher MIC values (Table 4) than another 6 or 7 isolates also obtained from farm A and B, respectively. The single isolate testing positive for *blaZ* solely differed in penicillin MIC values from the other 6 isolates obtained from farm C. Penicillin MIC values were strikingly different within the same dairy herd and ranged from ≤0.06 to 4 µg/mL (farms B and C, respectively) or from ≤0.06 to 32 µg/mL (farm A). The distribution of individual isolates from farms A, B, and C did not exceed more than 5 dilution levels. Concerning the remaining 9 dairy farms, the number of isolates varied from 1 to 18

Table 2. Distribution of MIC of *Staphylococcus aureus* (n = 93) isolated from 8 Danish dairy farms¹

Antimicrobial ²	MIC (µg/mL)										
	≤0.06	0.125	0.25	0.5	1	2	4	8	16	32	>32
Cefquinome	—	—	3	<i>55</i>	34	1	—	—	—	—	—
Cefoperazone	—	—	—	10	23	<i>54</i>	6	—	—	—	—
Cephapirin	16	28	<i>49</i>	—	—	—	—	—	—	—	—
Penicillin	88	3	2	—	—	—	—	—	—	—	—
Cloxacillin	—	18	<i>56</i>	18	1	—	—	—	—	—	—
Oxacillin	1	15	<i>47</i>	28	2	—	—	—	—	—	—
AMC	—	24	<i>63</i>	6	—	—	—	—	—	—	—
CFX-K	—	—	—	10	3	<i>71</i>	7	1	—	—	1

¹MIC₅₀ and MIC₉₀ are shown in italics and bold, respectively; bold italic indicates that MIC₅₀ and MIC₉₀ were identical.

²AMC = amoxicillin-clavulanic acid; CFX-K = cephalexin-kanamycin.

Table 3. Minimum inhibitory concentrations (MIC₅₀ and MIC₉₀) of German (n = 85) and Danish (n = 93) *Staphylococcus aureus* isolates

Antimicrobial	MIC (µg/mL)	Germany (n = 85)	Denmark (n = 93)
Cefquinome	MIC ₅₀	0.5	0.5
	MIC ₉₀	1	1
Cefoperazone	MIC ₅₀	2	2
	MIC ₉₀	4	2
Cephapirin	MIC ₅₀	0.125	0.25
	MIC ₉₀	0.5	0.25
Penicillin	MIC ₅₀	≤0.06	≤0.06
	MIC ₉₀	0.5	≤0.06
Cloxacillin	MIC ₅₀	0.25	0.25
	MIC ₉₀	1	0.5
Oxacillin	MIC ₅₀	0.25	0.25
	MIC ₉₀	1	0.5
Amoxicillin-clavulanic acid	MIC ₅₀	0.25	0.25
	MIC ₉₀	1	0.25
Cephalexin-kanamycin	MIC ₅₀	2	2
	MIC ₉₀	4	2

isolates per farm, and the distribution of isolates did not exceed 3 dilution levels of each antimicrobial agent.

Staphylococcus aureus Obtained from Danish Dairy Herds

The distribution of 93 Danish *S. aureus* isolates was narrow for all types of antimicrobials extending over 3 to 5 dilution levels except for cephalexin-kanamycin, because a single isolate had a MIC above the highest concentration tested for this combination (>32 µg/mL). The MIC₉₀ values were either one dilution level higher than MIC₅₀ values (cefquinome, cloxacillin, oxacillin) or MIC₅₀ and MIC₉₀ values corresponded to the same concentration level (cefoperazone, cephapirin, penicillin, amoxicillin-clavulanic acid, cephalexin-kanamycin). In total, 94.6% of Danish *S. aureus* had MIC values less or equal to the lowest penicillin concentration tested (≤0.06 µg/mL), representing the lowest MIC₅₀ and MIC₉₀ values. The highest MIC₅₀ and MIC₉₀ values were determined at a concentration of 2 µg/mL for cefoperazone and cephalexin-kanamycin, respectively. By

PCR, no isolate originating from Danish dairy farms was positive for the *mecA* or *blaZ* genes.

Staphylococcus aureus were randomly selected from 8 Danish dairy herds and the total number per farm varied from 2 to 50 isolates. The widest distribution pattern was apparent for cephalexin-kanamycin because of an isolate exhibiting a MIC of >32 µg/mL; the remaining 17 isolates from the same dairy herd had cephalexin-kanamycin MIC values between 0.5 and 2 µg/mL. Oxacillin MIC values of 50 *S. aureus* isolates obtained from 1 dairy herd showed a distribution ranging over at least 4 dilution levels (≤0.06 to 1 µg/mL). In the case of remaining β-lactams, distribution of individual isolates did not exceed more than 3 dilution levels, regardless of which dairy farm they originated from.

DISCUSSION

Studies on susceptibility testing of mastitis pathogens are rare in both Germany and Denmark. Furthermore, a variety of different methods are available for in vi-

Table 4. Minimum inhibitory concentrations (µg/mL) of *Staphylococcus aureus* isolates carrying *blaZ* and *mecA* (n = 5) and *blaZ* (n = 1) from 3 German dairy farms

Resistance gene	Dairy farm	MIC ¹ (µg/mL)							
		CEQ	CEFO	CPR	PEN	CLO	OXA	AMC	CFX-K
<i>blaZ</i> +, <i>mecA</i> +	A	1	16	2	16	2	8	2	4
	A	4	16	2	32	2	8	2	4
	A	2	16	4	16	1	4	2	4
	A	2	16	2	16	2	8	2	4
	B	0.5	8	1	4	0.5	1	2	4
<i>blaZ</i> +, <i>mecA</i> –	C	0.5	4	0.25	4	0.25	0.5	1	2

¹Antimicrobials: CEQ = cefquinome; CEFO = cefoperazone; CPR = cephapirin; PEN = penicillin; CLO = cloxacillin; OXA = oxacillin; AMC = amoxicillin-clavulanic acid; CFX-K = cephalexin-kanamycin.

tro susceptibility testing, which hinders comparison of these studies. However, the broth microdilution method is considered the gold standard for determining a pathogen's susceptibility (Constable and Morin, 2003).

Susceptibility Pattern of *S. aureus* Isolates from German Dairy Cows

Results of a questionnaire conducted between July 2001 and October 2002 on farms in northeastern Germany indicated that 35% of farms used third- (cefoperazone) and fourth- (cefquinome) generation cephalosporins for treating clinical mastitis, whereas oxacillin and cloxacillin were used in 17% and penicillin in 13% of all participating farms (Tenhagen et al., 2006).

The MIC₉₀ value of oxacillin investigated by Tenhagen and colleagues (2006) was 0.5 µg/mL, consistent with another German study (BVL, 2017). Even though distribution of isolates ranged from 0.06 to ≥16 µg/mL (BVL, 2017), the oxacillin MIC₉₀ in our study was one dilution level higher (1 µg/mL), in agreement with the study of De Oliveira et al. (2000).

Concerning amoxicillin-clavulanic acid, reported MIC₅₀ and MIC₉₀ values for *S. aureus* were 0.25 and 0.5 µg/mL, respectively (BVL, 2017). Tenhagen et al. (2006) found MIC₅₀ and MIC₉₀ values of 0.25 and 1 µg/mL; both MIC values corresponded with our results, even though amoxicillin-clavulanic acid was not used for treating clinical mastitis according to the questionnaire (Tenhagen et al., 2006). In contrast, MIC₅₀ and MIC₉₀ values of amoxicillin-clavulanic acid investigated by De Oliveira et al. (2000) were both ≤0.06 µg/mL.

Results of our study were in line with previous trials for MIC₅₀ and MIC₉₀ values of cefoperazone as well as cefquinome (Tenhagen et al., 2006; BVL, 2017). Moreover, MIC₉₀ values of *S. aureus* obtained from IMI were consistently 1 µg/mL in previous resistance monitoring programs (BVL, 2017). However, a German study investigating mastitis pathogens from cows with high SCC (>200,000 cells/mL) detected higher MIC values for cefquinome as well as cephapirin (Wente et al., 2016).

In accordance with our study, the highest and lowest MIC values among β-lactams tested against *S. aureus* were determined for penicillin (BVL, 2017). In this regard, penicillin MIC₅₀ was 0.03 µg/mL and MIC₉₀ was 16 µg/mL. In comparison to our study, the distribution of isolates investigated by the BVL (2017) was even wider and ranged from 0.015 to >64 µg/mL, whereby 5% of all isolates exhibited MIC values above the highest tested concentration (>64 µg/mL). Within the German resistance monitoring program in 2013, MIC₉₀ values of penicillin were 2 µg/mL (BVL, 2016), higher than determined for German *S. aureus*

in our study (0.5 µg/mL). When comparing results of 2013 and 2015, MIC₉₀ values of penicillin resulted in an 8-fold increase within 2 yr (BVL, 2016, 2017). Penicillin MIC₅₀ and MIC₉₀ values determined by De Oliveira et al. (2000) were coherent with results of German isolates investigated in our study.

Detection of *blaZ* and *mecA* in *S. aureus* from German Dairy Farms

In our study, resistance genes were detected in German *S. aureus* isolates only, from 3 dairy farms (Table 4). The detection of *blaZ* and *mecA* in 4 *S. aureus* isolates obtained from farm A was associated with comparatively higher MIC values. The 4 isolates that tested positive for both genes differed strongly in determined MIC values compared with 6 other isolates from farm A: cefquinome (≤1 µg/mL), cefoperazone (≤2 µg/mL), cephapirin (≤0.25 µg/mL), penicillin (≤0.125 µg/mL), cloxacillin (≤1 µg/mL), oxacillin (≤1 µg/mL), amoxicillin-clavulanic acid (≤0.25 µg/mL). On the one hand, resistance patterns might be spread vertically during multiplication of the bacterial cells. On the other hand, *blaZ* and *mecA* are located on mobile genetic elements and thus carry the risk of horizontal gene transfer between different bacteria (Schwarz and Chaslus-Dancla, 2001). Based on very different MIC values of *S. aureus* isolates from farm A leading to a bimodal distribution pattern, development of a resistant population could be assumed within the same dairy farm. However, increased MIC values might be present without existence of resistance genes; vice versa, the presence of resistance genes does not necessarily result in increased MIC values (EUCAST, 2018). Several isolates that were negative for *blaZ* and *mecA* were inhibited at equal or higher MIC values than the isolate obtained from farm B that was positive for both resistance genes (Table 1). A discrepancy between phenotypic and genotypic investigations was described in previous research studies (Haveri et al., 2005; Ruegg et al., 2015; Schmidt et al., 2015). One reason could be a mutation on the annealing side of the primer, which subsequently leads to resistance even in the absence of identified resistance genes. (Haveri et al., 2005; Schmidt et al., 2015). Another possible explanation might be the presence of resistance genes other than *mecA* that are associated with resistance to β-lactams. In this regard, the homologous resistance gene to *mecA*, *mecC* (formerly *mecA*_{LGA251}), has been detected in MRSA isolated from dairy cows. Although *mecC* is rarely described in German and Danish cows to date (Holmes and Zadoks, 2011; Schlotter et al., 2014; Hansen, 2018), the inclusion of *mecC* in future research projects could be important in MRSA surveillance. In

previous German studies, 3.3% (BVL, 2017) and 5.9% (BVL, 2016) of isolates carried *mecA*. In previous German studies, 3.3% (BVL, 2017) and 5.9% (BVL, 2016) of isolates carried *mecA*. This is in accordance with our study, since the number of *mecA*-positive bacteria also referred to individual isolates (5.9%). The occurrence of oxacillin-susceptible MRSA (OS-MRSA) was previously described on Chinese (Pu et al., 2014) and Brazilian dairy farms (Guimarães et al., 2017) and defined as phenotypes susceptible to oxacillin but carrying *mecA*. *Staphylococcus aureus* inhibited at oxacillin MIC >4 $\mu\text{g/mL}$ are classified as oxacillin resistant (Pu et al., 2014; Guimarães et al., 2017). In this context, the isolate originating from farm B (Table 4) could be assumed to be OS-MRSA, because the oxacillin MIC was 1 $\mu\text{g/mL}$, even though *mecA* was determined by PCR.

Spohr et al. (2011) determined MRSA in German dairy herds with variations between 3 dairy herds in Germany ranging from 5.1 to 16.7% (Spohr et al., 2011). Furthermore, Tenhagen et al. (2018) concluded an increase in the prevalence of MRSA on German dairy farms, because prevalence of MRSA was higher than previously described (Kreusukon et al., 2012; Tenhagen et al., 2014). Methicillin-resistant *S. aureus* in bulk tank milk was detectable on conventional and organic dairy farms at 9.7 and 1.7%, respectively (Tenhagen et al., 2018).

Susceptibility Pattern of *S. aureus* Isolates from Danish Dairy Farms

Chehabi et al. (2019) described the position of penicillin as the drug of first choice for mastitis treatment in Denmark as still being favorable. In their study, Chehabi et al. (2019) reported that 82.5% of all *S. aureus* isolates obtained from clinical mastitis were inhibited at penicillin MIC values of ≤ 0.06 $\mu\text{g/mL}$ (MIC₅₀), whereas the MIC₉₀ of penicillin was 2 $\mu\text{g/mL}$ (Chehabi et al., 2019). A previous Danish study in 2003 reported similar results: MIC₅₀ was 0.06 $\mu\text{g/mL}$ and MIC₉₀ was 4 $\mu\text{g/mL}$ (DANMAP, 2004). Both of these studies differed markedly from the MIC₉₀ of Danish *S. aureus* isolates in our study (Table 2). A study by Sato and colleagues (2004) examined *S. aureus* from bulk tank milk and reported lower MIC values. Both penicillin MIC₅₀ and MIC₉₀ of *S. aureus* from conventional farms were ≤ 0.06 $\mu\text{g/mL}$, whereas MIC₉₀ of *S. aureus* from organic farms was 0.25 $\mu\text{g/mL}$ (Sato et al., 2004). Moreover, Sato and colleagues (2004) reported that 100% of *S. aureus* isolates from both organic and conventional farms had oxacillin MIC values of 0.5 $\mu\text{g/mL}$ (Sato et al., 2004), which is consistent with our study findings. Results of De Oliveira et al. (2000) were also in line regarding oxacillin MIC values, whereas MIC₅₀ and MIC₉₀ values

of penicillin were ≤ 0.06 and 0.25 $\mu\text{g/mL}$, respectively (De Oliveira et al., 2000).

In Sweden, a country also following the Nordic guidelines, an overall restrictive antimicrobial use is pursued, and penicillin represented the drug of choice in 83% of all mastitis cases (Landin, 2007). Susceptibility testing of *S. aureus* showed that 92.9% of all isolates were inhibited at MIC ≤ 0.12 $\mu\text{g/mL}$ (Bengtsson et al., 2009). This is in agreement with MIC values of penicillin investigated in our study for Danish *S. aureus* isolates. The MIC₅₀ and MIC₉₀ values of oxacillin were 1 and 2 $\mu\text{g/mL}$, respectively (Bengtsson et al., 2009), 2 dilutions higher than those of the Danish isolates investigated in our study.

Prevalence of resistance genes has rarely been described in previous Danish studies on *S. aureus*. Genomic investigation of 63 *S. aureus* isolates from clinical mastitis cases identified 13 (20.6%) as positive for *blaZ* and 1 (1.6%) as positive for *mecA*. Of 94 *S. aureus* isolates from bulk tank milk, 14 (14.9%) were *blaZ* positive, and none were found to carry *mecA* (Ronco et al., 2018). Other Danish studies detected MRSA in bulk tank milk, with 2% *mecA*-positive isolates (DANMAP, 2013) and 3% consisting of 7 *mecA*-positive *S. aureus* isolates and one *mecC*-positive isolate (Hansen, 2018).

Susceptibility Patterns of German and Danish *S. aureus*: Country-Specific Antimicrobial Use

For at least half of the investigated isolates, country-specific differences in antimicrobial use were not evident because of equal MIC₅₀ values of most β -lactams. With the exception of cefquinome, German *S. aureus* isolates were inhibited at MIC₉₀ values at least one concentration level higher than Danish *S. aureus* isolates (Table 3).

Differences in phenotypic and genotypic investigations between German and Danish isolates in our study could be due to the generally restrictive use of antimicrobials in Denmark. Tenhagen et al. (2018) established a positive correlation between a strongly limited use of antimicrobial agents on organic farms and the appearance of resistance. In addition to the total number of MRSA isolates, antimicrobial resistance to most agents was even more pronounced in isolates obtained from conventional farms (Tenhagen et al., 2018). In contrast, Roesch et al. (2006) identified no significant differences in resistance between mastitis pathogens isolated from conventional and organic dairy farms. With regard to *S. aureus*, the prevalence of resistant isolates was higher from cows kept on organic farms (35%) compared with conventional farms (18%; Roesch et al., 2006).

In Germany in the first quarter of 2017, cefoperazone and cefquinome had market shares of 17% and 22%,

respectively, for the number of intramammary tubes sold. The most frequently used substance was cephalixin-kanamycin (27%), and the amoxicillin-clavulanic acid combination accounted for 19% (unpublished data provided by the Association for Consumer Research, GfK). Comparatively higher MIC₉₀ values of German *S. aureus* isolates against cefoperazone, amoxicillin-clavulanic acid, and cephalixin-kanamycin (Table 3) might result from their frequent use on German dairy farms. Selection advantages are considered the most important events associated with proliferation of pathogens resistant to certain antimicrobials (Barbosa and Levy, 2000).

However, results for cefquinome and penicillin contradict this hypothesis. Concerning cefquinome, neither MIC₅₀ nor MIC₉₀ values differed between German and Danish isolates, even though mastitis therapy in Denmark generally does not use cephalosporins (Landin et al., 2011; DANMAP, 2018). Moreover, the susceptibility of udder-associated *S. aureus* from German dairy farms to cephalosporins seems to have remained unaltered over the years (BVL, 2017), even though these substances have been used in mastitis treatment for more than 15 yr (Tenhagen et al., 2006). In contrast, penicillin is considered the drug of first choice for mastitis treatment in Denmark. Nonetheless, within our study, the MIC₉₀ of Danish *S. aureus* isolates was ≤ 0.06 µg/mL, substantially lower than the MIC₉₀ of German *S. aureus* isolates (0.5 µg/mL). Within the scope of resistance monitoring programs, penicillin MIC₉₀ increased to 16 µg/mL (BVL, 2017) by reducing penicillin use in mastitis therapy in Germany (Tenhagen et al., 2006; unpublished data provided by the Association for Consumer Research, GfK). Nevertheless, detection of 20.6% *blaZ*-positive isolates from clinical mastitis led to an assumption of higher penicillin resistance existing on Danish dairy farms (Ronco et al., 2018).

Previous studies examining the correlation between antimicrobial use and resistance of pathogens have drawn different conclusions. Saini et al. (2012) found a positive association between penicillin resistance in *S. aureus* and intramammary administered penicillin-novobiocin as well as penicillin that was administered systemically (Saini et al., 2012). Pol and Ruegg (2007) determined a correlation between antimicrobial exposure and susceptibility for some gram-positive pathogen-antimicrobial combinations only. Thus, *S. aureus* showed reduced susceptibility to penicillin treatment, whereas use of cephalixin did not result in decreased susceptibility, although it was also frequently used. Increased exposure of mastitis pathogens to most antimicrobial substances was not reflected in MIC values (Pol and Ruegg, 2007). Furthermore, Oliver et al. (2011) concluded in a review that routine use of antimicrobi-

als does not result in increased antimicrobial resistance in mastitis pathogens. Even though some drugs have been used for several decades, this has not led to a threatening resistance situation of mastitis pathogens (Oliver et al., 2011). Therefore, the effect on the pathogen's susceptibility and the development of resistance do not seem to depend only on the administered substance. Pathogen-specific properties associated with *S. aureus*, such as biofilm formation or the invasion of mammary epithelial cells, could additionally hamper the elimination of present pathogens and success of an antimicrobial therapy. Moreover, cows suffering from *S. aureus*-induced mastitis cases frequently become chronically infected (Barkema et al., 2006; Taponen and Pyörälä, 2009; Rainard et al., 2018). Wente et al. (2016) assumed that increased MIC values might be due to the tendency toward chronicity of subclinical mastitis. Dairy cows exhibiting a high SCC might therefore undergo antimicrobial treatment more frequently. Danish *S. aureus* isolates investigated in the current study contradict the assumption of higher MIC values associated with pathogens obtained from subclinical mastitis cases. Oliveira et al. (2012) did not make a general statement about susceptibility of *S. aureus* from clinical or subclinical mastitis cases due to strong variations in MIC values of isolates regardless of their particular origin (Oliveira et al., 2012). A more likely factor influencing the development of resistance might be the frequency of antimicrobial treatment. This is also in line with investigations by Rajala-Schultz et al. (2004), who detected higher resistance in older cows, attributing it to increased exposure to antimicrobial treatment. Furthermore, because Denmark generally limits antimicrobial use in mastitis therapy as much as possible, this likely explains the lower MIC values of Danish *S. aureus* from subclinical mastitis cases. Nevertheless, further research focusing on the relation between antimicrobial use and occurrence of resistance would be a meaningful approach to detect relevant influencing factors.

Recommendations for therapy derived from susceptibility testing of pathogens are usually carried out based on specific breakpoints used for classifying isolates into clinically resistant, intermediate, and susceptible. According to clinical breakpoints previously used to categorize *S. aureus* from IMI (Thomas et al., 2015; de Jong et al., 2018; Käppeli et al., 2019), 2 (2.2%) and 1 (1.1%) Danish *S. aureus* isolates could be classified as resistant to penicillin and cephalixin-kanamycin, respectively. Furthermore, the breakpoints indicated low resistance of German *S. aureus* isolates to penicillin (11.8%), oxacillin (4.7%), cloxacillin (0%), cephalixin (0%) amoxicillin-clavulanic acid (0%), and cephalixin-kanamycin (0%). From this point of view, we cannot

establish an urgent need to use critically important cephalosporins, because various antimicrobial agents are appropriate alternatives. However, the validity of this statement is limited by several aspects that need to be considered in the discussion of resistance of mastitis pathogens. First, methodology and interpretative criteria need to follow the same susceptibility testing guidelines for an accurate assessment (Schwarz et al., 2010). Moreover, clinical breakpoints for cefquinome and cefoperazone are still lacking, and breakpoints for the remaining β -lactams are not specific to mastitis. The Clinical and Laboratory Standards Institute (CLSI) has published clinical breakpoints for 3 antimicrobial agents related to mastitis: ceftiofur, penicillin-novobiocin, and pirlimycin (Ruegg et al., 2015). These antimicrobial agents have either not been approved for intramammary therapy or are not relevant in mastitis treatment in Germany or Denmark (DANMAP 2018; Wallmann et al., 2018; unpublished data provided by the Association for Consumer Research, GfK). A lack of specific clinical breakpoints for mastitis leads to the transfer of breakpoints established for different indications, for other animal species, or in human medicine. The statement concerning the present resistance situation, and especially recommendations for therapy resulting from such a classification, is questionable. Haveri et al. (2005) hypothesized that discrepancies in genotypic and phenotypic investigations could be due to the use of improper clinical breakpoints. This problem was largely avoided in our study by focusing on MIC values and distribution patterns, rather than breakpoints. The dosage achieved at the site of infection varies depending on the tissue and does not reflect the concentration reached in the mammary gland. For instance, β -lactams achieve a high concentration in the urine, and breakpoints based on urinary tract infections might thus be set higher. Transmission of those breakpoints to mastitis pathogens is inappropriate and can lead to misinterpretations (Rossitto et al., 2002; Schwarz et al., 2010). Therefore, we emphasize the need to establish internationally standardized clinical breakpoints to adequately assess the resistance situation and derive effective recommendations for mastitis therapy.

CONCLUSIONS

The German *S. aureus* isolates investigated in the present study were inhibited at comparatively higher MIC₉₀ values for most β -lactams than isolates from Danish dairy farms. The *blaZ* and *mecA* resistance genes were isolated in individual German *S. aureus* isolates only and were generally associated with comparatively higher MIC values. The predominant use of penicillin in Denmark and of third- (cefoperazone) and fourth-

(cefquinome) generation cephalosporins in Germany was not directly reflected in determined MIC values. Results led to the conclusion that development of resistance is not solely dependent on the administered substance. Further research focusing on the interactions of antimicrobial use and development of resistance would be appropriate for determining decisive influencing factors and for establishing suitable preventive measures. Therapy recommendations deriving from results of this study are not directly transferable to in vivo practice or derived therapy recommendations, highlighting the need for internationally standardized clinical breakpoints.

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