Interpretive Summary

Bolte

Staphylococcus aureus is a common mastitis pathogen in Germany and Denmark. In the present study, 85 and 93 *S. aureus* isolates from 12 German and 8 Danish dairy farms were tested for their susceptibility to 8 antimicrobials. Thus, MIC values were determined for each isolate, followed by PCR methods for detecting resistance genes (*bla*Z, *mec*A). Danish *S. aureus* isolates exhibited generally lower MIC_{90} values concerning most tested β-lactams. A total of 5 German isolates carried both resistance genes and one additional isolate carried *bla*Z only. A correlation between predominantly used antimicrobials and reduced susceptibility could not be established.

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

J. Bolte[1], Y. Zhang[1], N. Wente[1], Y. Mahmmod[2,3,4], L. Svennesen[5], V. Krömker[1,5]*

[1] Department of Microbiology, University of Applied Sciences and Arts, D-30453 Hannover, Germany

[2] Centre de Recerca en Sanitat Animal (CReSA, IRTA), Universitat Autònoma de Barcelona, Bellaterra, 08193, Barcelona, Spain

[3] Veterinary Sciences Division, Al Ain Men's College, Higher Colleges of Technology, 17155- Al Ain, Abu Dhabi, United Arab Emirates.

[4] Infectious Diseases Division, Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, 44511-Sharkia, Egypt

[5] Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, DK-1870 Frederiksberg C, Denmark

*corresponding author:
Prof. Dr. Volker Krömker
Department of Microbiology, University of Applied Sciences and Arts, D-30453 Hannover, Germany.
Tel.
Fax.
Email: Volker.Kroemker@hs-hannover.de

ABSTRACT

Staphylococcus (S.) aureus is one of the most common pathogens associated with bovine mastitis in Germany as well as Denmark. A successful therapy is, among others, 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 of 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 *bla*Z and *mec*A resistance genes via PCR methods. The tested antimicrobials refer to the most frequently used β-lactams in German and Danish dairy farms including cefquinome, cefoperazone, cephapirin, penicillin, oxacillin, cloxacillin, amoxicillin / clavulanic acid and cefalexin / kanamycin. Special attention was paid to varying therapy concepts, since in Germany, third and fourth generation cephalosporins have been predominantly used in mastitis therapy so far, whereas in Denmark, restrictive use of penicillin is pursued by a general avoidance of cephalosporins. Differences in MIC values were mainly based on determined MIC_{90} values. In general, Danish *S. aureus* isolates were inhibited at comparatively lower MIC_{90} values than *S. aureus* isolated from German dairy farms for most β-lactams. No differences could be observed regarding cefquinome, since both German and Danish isolates exhibited MIC_{50} and MIC_{90} values of 0.5 µg/mL and 1 µg/mL, respectively. In contrast, penicillin MIC_{90} against German and Danish *S. aureus* was 0.5 µg/mL and ≤ 0.06 µg/mL, respectively. Resistance genes (*bla*Z, *mec*A) were only detected in German *S. aureus* isolates on three dairy farms in Germany. A total of 5 isolates were tested positive for both *bla*Z and *mec*A, whereas an individual isolate carried the *bla*Z resistance gene only. A direct correlation between frequently used antimicrobials and reduced susceptibility could not be determined on the basis of the present study. Besides further research for determining fac-

tors associated with resistance development, we emphasize the urgent need for internationally standardized clinical breakpoints for assessing resistance situations more accurately.

Keywords: *Staphylococcus aureus*, mastitis, minimum inhibitory concentrations, *bla*Z, *mec*A

INTRODUCTION

In dairy farming, *Staphylococcus* (*S.*) *aureus* plays an important role as a major pathogen of clinical as well as subclinical mastitis. The 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). Resistances to these β-lactams are mainly based on two different resistance mechanisms. One is encoded by the *bla*Z resistance gene and acts via an enzymatic inactivation by β-lactamases. These β-lactamases are capable of attacking the β-lactam ring by hydrolysis, resulting in inactive metabolites. The other one is encoded by the *mec*A gene and 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, 2001; Wendlandt et al., 2013). A mutual transmission of MRSA between dairy cows and humans, especially those standing in close contact to animals, have further been 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 as posing a higher risk to human health than natural penicillins and narrow-spectrum penicillins (category 1) (EMA, 2014). In Germany, the total amount of antimicrobial agents dispensed in veterinary medicine decreased by 972.6 tons (57 %) between 2011 and 2017. Whereas the whole group of penicillins decreased by 258.9 tons, 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)). Since March 2018, susceptibility testing is required prior to all cases of treatment with third and fourth generation cephalosporins (BMJV, 2018).

In contrast, Nordic countries such as Denmark, Norway and Sweden pursue a restrictive antimicrobial use by avoiding cephalosporins as far as possible. Thus, treatment of *S. aureus* with β-lactamase resistant antimicrobials is just 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 should be 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 bacterial diagnosis beforehand (Carmo et al., 2017). Denmark thus reduced its overall antimicrobial intake by more than 16 tons between 2013 and 2017 and continuously shifted away from the use of cephalosporins. Intramammary antimicrobial therapy is performed by a predominant use of β-lactamase sensitive penicillins and represented 59.8 % of all antimicrobials used for intramammary treatment in 2017. The β-lactamase stable penicillins like oxacillin and extended-spectrum penicillins including combinations with β-lactamase inhibitors made up 20.3 % and the cephalosporins 10.5 % of the total. Regarding the latter, the relation of first to third and fourth generation cephalosporins for intramammary application differed with a percentage of 96.6 % to 3.4 % (DANMAP, 2018).

Due to an obviously contrasting antimicrobial use in mastitis therapy in Germany and Denmark, the objective of the present study was to identify whether this is reflected in different susceptibility patterns of *S. aureus* as well as considering the presence of *bla*Z and *mec*A resistance genes.

MATERIALS AND METHODS

Collection of milk samples and microbiological identification of isolates

In the present study, a total of 178 *S. aureus* isolates from intramammary infections of dairy cows were used for susceptibility testing and genotypic investigations. German and Danish farms were representatives of milk producing dairy farms of the respective countries with a predominant use of either cephalosporins of third and fourth generation (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 were send to the microbiological laboratory for routine diagnosis. Milk samples were therefore 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 1325 cows and the Holstein Friesian breed was predominantly kept on Northern German dairy farms. The annual milk production ranged from 8726 to 12353 kg ECM per cow and a bulk tank SCC varying 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 containing boric acid (Ly20) as a preservative. Immediately after sampling, tubes were cooled below 6 °C and sent to the microbiological laboratory of the University of Applied Sciences and Arts (Hannover, Germany) for etiological diagnosis. Examinations of samples including cultivation and identification of pathogens were conducted in accordance with the methods described by the German Veterinary Medical Association (2009). A quantity of 10 µL of each milk sample was plated on a quadrant of esculin blood agar (Oxoid Deutschland GmbH, Germany) and incubated at 37 °C under aerobic conditions. After 24 and 48 hours of incubation, grown colonies were first investigated concerning their morphology and hemolysis patterns. Besides gram staining, examination of cell morphology and catalase activities (3 % H_2O_2; Merck AG, Germany), the clumping factor test

(DiaMondiaL Staph Plus Kit, Virotech Diagnostics GmbH, Germany) serves for an initial differentiation of presumptive *S. aureus* isolates. The detection of the *S. aureus*-specific *nuc* gene was performed in accordance with Saiful et al. (2006) and finally confirmed the presence of *S. aureus* in milk samples of intramammary infections (Saiful et al., 2006).

A total of 93 *S. aureus* isolates originating from milk samples of dairy cows with subclinical mastitis (≥ 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 within the period of February 2017 to April 2017 by project researchers of the University of Copenhagen (Denmark). Between 30 and 40 dairy cows with an elevated SCC (> 200,000 cells/mL), but lacking of 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 ECM per cow and a bulk tank SCC ranging from 183,000 to 338,000 cells/mL. Danish Holstein was the predominant breed of all dairy farms, whereby two herds were entirely consisting of Danish Holstein. Sample collection and identification of *S. aureus* including bacterial culturing as well as MALDI-TOF assay were performed and previously described by the project researchers (Mahmmod et al., 2018; Svennesen et al., 2019). Out of the total number of the harvested *S. aureus* isolates from the projects, 93 isolates were randomly selected to be included in the current study. After identifying all pathogens as *S. aureus*, both German as well as 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 Minimal Inhibitory Concentration (MIC) is a measurement of a pathogen's susceptibility to an antimicrobial agent and 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 by scientific collaborators of the University of Applied Sciences and Arts in Hannover (Germany). The tested antimicrobial agents were selected on the basis of 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 cefalexin / kanamycin (2:1). The proportion of the tested combinations (amoxicillin / clavulanic acid, cefalexin / kanamycin) referred 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, Germany) were used containing a 2-fold dilution series of each antimicrobial substance ranging from 0.06 µg/mL to 32 µg/mL.

Respective concentration levels were prepared by using Mueller-Hinton-Bouillon (Carl Roth GmbH, Germany) as a liquid nutrient medium. Wells of the microtiter plates finally contained 100 µL of an antimicrobial solution to which 5 µL of an immediately prepared bacterial inoculum suspension were added. Positive controls (wells only containing Mueller-Hinton-Bouillon) 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, Germany) was used as a reference strain. After incubating the microtiter plates for 16 to 20 hours at 37 °C, MIC were determinable as wells containing no visible turbidity corresponding to the lowest concentration that inhibits bacterial growth.

Determination of present blaZ and mecA resistance genes

Extraction of the bacterial DNA was performed by using the DNeasy Blood & Tissue Kit (QIAGEN N.V., the Netherlands). The PCR was carried out in 25 µL reaction mix including 12.5 µL ReadyMix Taq PCR Reaction Mix (Sigma-Aldrich GmbH, Germany), 20 pmol of primer, 5 µL of the template and H_2O for no template control. Amplification reactions were performed in a Stratagene Mx3005P qPCR System Thermocycler (Agilent Technologies Inc., United States). Primers used for detecting resistance genes as well as temperature profiles were programed as previously described for *blaZ* (Vesterholm-Nielsen et al., 1999) and *mec*A (Saiful et al., 2006). The PCR products were directly stained with Midori Green Direct (Nippon Genetics Europe GmbH, Germany) and separated in a 2 % agarose gel at 100 V for 2 hours.

RESULTS

Results of MIC examinations for *S. aureus* isolates obtained from intramammary infections of dairy cows are presented in detail in Table 1 and Table 2 for 85 German and 93 Danish *S. aureus* isolates, respectively. Besides the distribution of isolates according to determined MIC values, MIC_{50} and MIC_{90} are given as well (Table 1 and Table 2). MIC_{50} and MIC_{90} are defined as the lowest concentration where growth of at least 50 % and 90 % of the tested bacteria are inhibited, respectively (DIN, 2007). A direct comparison of MIC_{50} and MIC_{90} values of German and Danish *S. aureus* isolates is additionally presented in Table 3.

S. aureus obtained from German dairy herds

Due to determined MIC values, the distribution of 85 tested German *S. aureus* isolates was wide for most β-lactams and extended over at least 5 dilution levels with the exception of cefalexin / kanamycin ranging from 1 µg/mL to 4 µg/mL. The widest distribution pattern was

236 determined for penicillin ranging from ≤ 0.06 mg/mL to 32 mg/mL, without an inhibition of
237 isolates at concentration levels of 1 µg/mL, 2 µg/mL and 8 µg/mL. The highest MIC_{50} and
238 MIC_{90} values were 2 µg/mL and 4 µg/mL, respectively for cefoperazone as well as for the
239 combination of cefalexin / kanamycin. MIC_{90} values of cefquinome, cefoperazone and ce-
240 falexin / kanamycin were one dilution higher than their MIC_{50} values, whereas MIC_{90} of
241 cephapirin, cloxacillin, oxacillin and amoxicillin / clavulanic acid were 2 dilutions higher than
242 their MIC_{50} values. The lowest MIC_{50} value was determined for penicillin, whereas MIC_{90}
243 values (0.5 µg/mL) were lowest for penicillin as well as cephapirin. Since the MIC_{50} value of
244 penicillin was determined as ≤ 0.06 µg/mL, the penicillin MIC_{90} value was at least 3 dilutions
245 higher (Table 1).
246 Within the PCR examination, a total of 5 isolates (5.9 %) were found to be positive for carry-
247 ing *mec*A as well as *bla*Z. Out of these, 4 isolates originated from the same dairy farm (Farm
248 A), whereas a single isolate carrying both resistance genes was isolated from another dairy
249 farm in northern Germany (Farm B). Besides these 5 isolates positive for both *mec*A and
250 *bla*Z, an additional isolate originating from a different farm (Farm C) was identified as posi-
251 tive for *bla*Z only (Table 4).
252 The distribution patterns of isolates originating from farm A and B extended over several di-
253 lution levels of the tested β-lactams. This was based on isolates that carried resistance genes
254 and were inhibited at higher MIC values than another 6 or 7 isolates also obtained from farm
255 A and B, respectively. The single isolate tested positive for *bla*Z solely differed in penicillin
256 MIC values from 6 isolates obtained from farm C as well. Penicillin MIC values were striking
257 different within the same dairy herd and ranged from ≤ 0.06 µg/mL to 4 µg/mL (Farm B and
258 C, respectively) or even ≤ 0.06 µg/mL to 32 µg/mL (farm A). The distribution of individual
259 isolates received from farm A, B and C did not exceed more than 5 dilution levels. Concern-
260 ing the remaining 9 dairy farms, the number of isolates varied from 1 to 18 isolates per farm,

whereby the distribution of isolates did not exceed 3 dilution levels of each antimicrobial agent.

S. aureus obtained from Danish dairy herds

The distribution of 93 Danish *S. aureus* isolates was narrow for all kinds of antimicrobials extending over 3 to 5 dilution levels except for cefalexin / kanamycin, since a single isolate exhibited a MIC above the highest concentration tested for this combination (> 32 µg/mL). MIC_{90} values were either one dilution level higher than MIC_{50} values (cefquinome, cloxacillin, oxacillin) or MIC_{50} and MIC_{90} values corresponded to the same concentration level (cefoperazone, cephapirin, penicillin, amoxicillin / clavulanic acid, cefalexin / 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 MIC_{50} as well as MIC_{90} values. Highest MIC_{50} and MIC_{90} values were determined at a concentration level of 2 µg/mL for cefoperazone and cefalexin / kanamycin, respectively.

Within genotypic investigations, no isolate originating from Danish dairy farms was positive for carrying *mec*A or *bla*Z resistance genes.

S. aureus were randomly selected from 8 Danish dairy herds and the total amount per farm varied from 2 to 50 isolates. The widest distribution pattern was apparent for cephalexin / kanamycin due to the *S. aureus* isolate exhibiting a MIC of > 32 µg/mL, whereas the remaining 17 isolates obtained from the same dairy herd had cefalexin / kanamycin MIC values between 0.5 µg/mL and 2 µg/mL. Oxacillin MIC values of 50 *S. aureus* isolates obtained from one dairy herd showed a distribution ranging over at least 4 dilution levels (≤ 0.06 µg/mL 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 quite rare in both Germany and Denmark. Furthermore, a variety of different methods are available for in vitro susceptibility testing which hinders comparison of these studies. However, the broth microdilution method is considered as the "gold standard" for determining the 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 nort-eastern Germany indicated that 35 % of farms used cephalosporins of third (cefoperazone) and fourth (cefquinome) generation for treating clinical mastitis, whereas oxacillin and cloxa-cillin were used in 17 % and penicillin in 13 % of all participating farms (Tenhagen et al. 2006).

The MIC_{90} value of oxacillin investigated by Tenhagen and colleagues (2006) was 0.5 µg/mL and is consistent with another German study (BVL, 2017). Even though distribution of iso-lates ranged from 0.06 µg/mL to ≥ 16 µg/mL (BVL, 2017), oxacillin MIC_{90} in our study was one dilution level higher (1 µg/mL) and in agreement with the study of De Oliveira et al. (2000).

Concerning amoxicillin / clavulanic acid, MIC_{50} and MIC_{90} values of *S. aureus* were 0.25 µg/mL and 0.5 µg/mL, respectively (BVL, 2017). Tenhagen et al. (2006) found MIC_{50} and MIC_{90} values of 0.25 µg/mL and 1 µg/mL and thus 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, MIC_{50} and MIC_{90} values of amoxicillin / clavulanic acid investigated by De Oliver et al. (2000) were both ≤ 0.06 µg/mL.

312 Results of our study were in line with previous trials for both MIC_{50} and MIC_{90} values of
313 cefoperazone as well as cefquinome (Tenhagen et al., 2006; BVL, 2017). Moreover, MIC_{90}
314 values of *S. aureus* obtained from intramammary infections were constantly at 1 µg/mL with-
315 in previous resistance monitoring programs (BVL, 2017). However, a German study investi-
316 gating mastitis pathogens from cows with high SCC (> 200,000 cells/mL) detected higher
317 MIC values for cefquinome as well as cephapirin (Wente et al., 2016).
318 In accordance with our study, highest and lowest MIC values among β-lactams tested against
319 *S. aureus* were determined for penicillin (BVL, 2017). In this regard, penicillin MIC_{50} was
320 0.03 µg/mL, whereas MIC_{90} of *S. aureus* was 16 µg/mL. Moreover, the distribution of isolates
321 was even wider than examined in our study and ranged from 0.015 µg/mL to > 64 µg/mL,
322 whereby 5 % of all isolates exhibited MIC values above the highest tested concentration (> 64
323 µg/mL). Within the German resistance monitoring program in 2013, MIC_{90} values of penicil-
324 lin were 2 µg/mL (BVL, 2016) and thus higher than determined for German *S. aureus* in our
325 study (0.5 µg/mL). When comparing results of 2013 and 2015, MIC_{90} values of penicillin
326 resulted in an 8-fold increase within two years (BVL, 2016; BVL, 2017). Penicillin MIC_{50}
327 and MIC_{90} values determined by De Oliveira et al. (2000) were coherent with results of Ger-
328 man isolates investigated in our study.
329
330 ***Detection of blaZ and mecA resistance genes in S. aureus from German dairy farms***
331 In our study, resistance genes were detected in German *S. aureus* isolates only, referring to 3
332 dairy farms in Germany (Table 4). The detection of *blaZ* and *mec*A in 4 *S. aureus* isolates
333 obtained from farm A is associated with comparatively higher MIC values. The 4 isolates,
334 which were tested positive for both resistance genes, differed strongly concerning determined
335 MIC values from an additional 6 isolates obtained from Farm A as well: cefquinome (≤ 1
336 µg/mL), cefoperazone (≤ 2 µg/mL), cephapirin (≤ 0.25 µg/mL), penicillin (≤ 0.125 µg/mL),
337 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 replication of the bacterial cells. Additionally, *blaZ* as well as *mec*A are located on mobile genetic elements and thus harbour the risk of a horizontal gene transfer between different bacteria (Schwarz and Chaslus-Dancla, 2001). Based on strongly 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 or vice versa the presence of resistance genes do not necessarily result in increased MIC values (EUCAST, 2018). Several isolates tested negative for *blaZ* and *mec*A were inhibited at equal or higher MIC values than that isolate obtained from farm B tested positive for both resistance genes (Table 1). A discrepancy between phenotypic and genotypic investigations was already 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 the occurrence of resistances in the absence of identified resistance genes. (Haveri et al., 2005; Schmidt et al. 2015). Another possible explanation might be the presence of additional resistance genes other than *mec*A, which are associated with resistances to β-lactams. In this regard, the *mec*A homologous resistance gene *mec*C (former *mec*A$_{LGA251}$) could already be detected in MRSA isolated from dairy cows. Although *mec*C is rarely described in German and Danish cows to date (Holmes and Zadoks, 2011; Schlotter et al., 2014; Hansen, 2018), the inclusion of the *mec*C resistance gene in prospective research projects could be important in the future MRSA surveillance. In previous German studies, 3.3 % (BVL, 2017) and 5.9 % (BVL, 2016) were identified as carrying *mec*A. In comparison to our study (5.9 %), occurrence of *mec*A positive bacteria only referred to individual isolates as well. The occurrence of oxacillin susceptible MRSA (OS-MRSA) was previously described on Chinese (Pu et al., 2014) and Brazilian dairy farms (Guimaraes et al., 2017) and was defined as phenotypical susceptible to oxacillin but carrying *mec*A. *S. aureus* inhibited at oxacillin MIC greater than 4 µg/mL were classified as oxacillin

364 resistant (Pu et al., 2014; Guimaraes et al., 2017). In this context, the isolate originating from
365 farm B (Table 4) can thus be assumed as OS-MRSA, since oxacillin MIC was 1 µg/mL even
366 though the appearance of *mec*A was determined within PCR examination.
367 Spohr et al. (2011) determined MRSA in German dairy herds with variations between three
368 dairy herds in Germany ranging from 5.1 – 16.7 % (Spohr et al., 2011). Furthermore, Tenha-
369 gen et al. (2018) concluded an increase in the prevalence of MRSA on German dairy farms,
370 since prevalence of MRSA was higher than previously described (Kreausukon et al., 2012;
371 Tenhagen et al., 2014). MRSA in bulk tank milk was thus detectable on conventional and
372 organic dairy farms with 9.7 % and 1.7 %, respectively (Tenhagen et al., 2018).
373
374 ***Susceptibility pattern of S. aureus isolates from Danish dairy farms***
375 Chehabi et al. (2019) currently described the position for penicillin as drug of first choice for
376 mastitis treatment in Denmark as still being favorable. In their study, Chehabi et al. (2019)
377 reported that 82.5 % of all *S. aureus* isolates obtained from clinical mastitis were inhibited at
378 penicillin MIC values of ≤ 0.06 µg/mL (MIC_{50}), while MIC_{90} of penicillin was determined as
379 2 µg/mL (Chehabi et al., 2019). A previous Danish study in 2003 came to similar results as
380 MIC_{50} was 0.06 µg/mL and MIC_{90} was 4 µg/mL (DANMAP, 2004). Both of these studies
381 differed markedly with regard to results for MIC_{90} of Danish *S. aureus* isolates in our study
382 (Table 2). A study by Sato and colleagues (2004) examined *S. aureus* from bulk tank milk
383 resulting in lower MIC values. Both penicillin MIC_{50} as well as MIC_{90} of *S. aureus* from con-
384 ventional farms were ≤ 0.06 µg/mL, whereas MIC_{90} of *S. aureus* from organic farms was 0.25
385 µg/mL (Sato et al., 2004). Moreover, Sato and colleagues (2004) investigated 100 % of *S.*
386 *aureus* isolates from both organic and conventional farms having oxacillin MIC values of 0.5
387 µg/mL (Sato et al., 2004) which is consistent with our study findings. Results of De Oliveira
388 et al. (2000) were also in line regarding oxacillin MIC values, whereas MIC_{50} and MIC_{90}

values of penicillin were ≤ 0.06 µg/mL and 0.25 µg/mL, respectively (De Oliveira et al., 2000).

In Sweden, a country also following the Nordic guidelines for mastitis therapy, restrictive use of preferable penicillin was used in 83 % of all mastitis cases (Landin, 2007) and susceptibility testing of *S. aureus* identified that 92.9 % of all isolates were inhibited at MIC ≤ 0.12 µg/mL (Bengtsson et al., 2009). This is in agreement with MIC values of penicillin investigated in our study for Danish *S. aureus* isolates. MIC_{50} and MIC_{90} values of oxacillin were 1 µg/mL and 2 µg/mL, respectively (Bengtsson et al., 2009) and thus 2 dilutions higher than those from Danish isolates investigated in our study.

Prevalence of resistance genes was rarely described in previous Danish studies on *S. aureus*. Genomic investigation of 63 *S. aureus* isolates from clinical mastitis cases identified 13 (20.6 %) as carrying *bla*Z and 1 (1.6 %) as positive for *mec*A. Of the 94 *S. aureus* isolates from bulk tank milk, 14 (14.9 %) were *bla*Z positive, whereas none (0 %) were determined as carrying *mec*A (Ronco et al., 2018). Other Danish studies detected MRSA in bulk tank milk to the amount of 2 % as *mec*A positive isolates (DANMAP, 2013) and 3 % consisting of seven *mec*A positive *S. aureus* isolates and one *mec*C positive one (Hansen, 2018).

Susceptibility patterns of German and Danish S. aureus with regard to country-specific antimicrobial use

For at least half of the investigated isolates, country-specific differences in antimicrobial use were not evident due to equal MIC_{50} values of most β-Lactams. With the exception of cefquinome, German *S. aureus* isolates were inhibited at MIC_{90} values that were at least 1 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) were already able to establish a positive correlation between a strongly

limited use of antimicrobial agents on organic farms and the appearance of resistance. Besides 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 even higher in isolates obtained from cows kept on organic farms (35 %) than those obtained from 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 concerning the number of intramammary tubes sold. The most frequently used substance was cefalexin / kanamycin (27 %), whereas the combination of amoxicillin / clavulanic acid accounted for 19 % (unpublished data provided by the Association for Consumer Research (GfK)). Comparatively higher MIC_{90} values of German *S. aureus* isolates against cefoperazone, amoxicillin / clavulanic acid and cefalexin / kanamycin (Table 3) might thus result from a frequent use on German dairy farms. Selection advantages are considered as the most important events associated with the proliferation of pathogens resistant to certain antimicrobials (Barbosa and Levy, 2000).

However, results for cefquinome and penicillin contradict this hypothesis. Concerning cefquinome, neither MIC_{50} nor MIC_{90} values differed between German and Danish isolates, even though mastitis therapy in Denmark generally refrains from using cephalosporins (Landin, 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), regardless of the fact that these substances were predominantly used in mastitis treatment for more than 15 years (Tenhagen et al., 2006). On the other hand, penicillin is considered as the drug of first choice for mastitis treatment in Denmark. Nonetheless, within our study, MIC_{90} of Danish *S. aureus* isolates was determined as ≤ 0.06 µg/mL and was thus substantially lower than MIC_{90} of German *S. aureus* isolates (0.5 µg/mL). Within the scope of

441 resistance monitoring programmes, penicillin MIC_{90} increased to 16 µg/mL (BVL, 2017) by
442 simultaneously reducing penicillin use in mastitis therapy in Germany (Tenhagen et al., 2006;
443 unpublished data provided by the Association for Consumer Research (GfK)). Nevertheless,
444 detection of 20.6 % *bla*Z positive isolates led to the assumption of even higher penicillin re-
445 sistance existing on Danish dairy farms (Ronco et al., 2018).
446 Previous studies examining the correlation between antimicrobial use and resistance of patho-
447 gens draw different conclusions. Saini et al. (2012) found a positive association between pen-
448 icillin resistance in *S. aureus* and intramammary administered penicillin / novobiocin as well
449 as penicillin that was administered systemically (Saini et al., 2012). Pol and Ruegg (2007)
450 determined a correlation between antimicrobial exposure and susceptibility for some Gram-
451 positive pathogen-antimicrobial combinations only. Thus, *S. aureus* showed a reduced sus-
452 ceptibility to penicillin treatment, whereas use of cephapirin did not result in decreased sus-
453 ceptibility, although it was frequently used as well. An increased exposure of mastitis patho-
454 gens to most antimicrobial substances was not reflected in MIC values (Pol and Ruegg, 2007).
455 Furthermore, Oliver et al. (2011) concluded in a review that routinely used antimicrobials do
456 not result in increased antimicrobial resistance in mastitis pathogens. Even though the fact
457 some drugs have been used for several decades did not lead to a threatening resistance situa-
458 tion of mastitis pathogens (Oliver et al., 2011). Therefore, the influence on the pathogen's
459 susceptibility and the development of resistance seems not to be only dependent on the ad-
460 ministered substance. Pathogen-specific properties associated with *S. aureus,* such as biofilm
461 formation and/ or the invasion of mammary epithelia cells, could additionally hamper the
462 elimination of present pathogens and success of an antimicrobial therapy. Moreover, cows
463 suffering from *S. aureus* induced mastitis cases become frequently chronically infected
464 (Barkema et al., 2006; Taponen and Pyörälä 2009, Rainard et al., 2017). Wente et al. (2016)
465 assumed that increased MIC values might be due to the tendency towards chronicity of sub-
466 clinical mastitis. Dairy cows exhibiting a high SCC might therefore undergo antimicrobial

467 treatment more frequently. Danish *S. aureus* isolates investigated in the current study contra-
468 dict from the assumption of higher MIC values associated with pathogens obtained from sub-
469 clinical mastitis cases. Additionally, results of Oliveira et al. (2012) did also not provide a
470 general statement about susceptibility of *S. aureus* from clinical or subclinical mastitis cases
471 due to strong variations in MIC values of isolates, regardless of their particular origin
472 (Oliveira et al., 2012). A more likely influencing factor for the development of resistances
473 might be the frequency of antimicrobial treatments. This is also in line with investigations of
474 Rajala-Schultz et al. (2004), which detected higher resistances in older cows and attributed
475 this to an increased exposure to antimicrobial treatments (Rajala-Schultz et al., 2004). Fur-
476 thermore, since Denmark generally limits the antimicrobial use in mastitis therapy as much as
477 possible, this could be the probable explanation for lower MIC values of Danish *S. aureus*
478 from subclinical mastitis cases. Nevertheless, further research focusing on the relation be-
479 tween antimicrobial use and occurrence of resistance would be a meaningful tool in the future
480 for detecting relevant influencing factors.
481 Recommendations for therapy derived from susceptibility testing of pathogens are usually
482 carried out on the basis of specific breakpoints used for classifying isolates into clinically re-
483 sistant, intermediate and susceptible. According to clinical breakpoints previously used to
484 categorize *S. aureus* from intramammary infections (Thomas et al., 2015; De Jong et al.,
485 2018; Käppeli et al., 2019), two (2.2 %) and another single (1.1 %) Danish *S. aureus* could be
486 classified as resistant to penicillin and cefalexin / kanamycin, respectively. Furthermore, the
487 breakpoints indicate low resistances of German *S. aureus* isolates to penicillin (11.8 %), oxa-
488 cillin (4.7 %), cloxacillin (0 %), cephapirin (0 %) amoxicillin / clavulanic acid (0 %) and ce-
489 falexin / kanamycin (0 %). From this point of view, an urgent need for using critically im-
490 portant cephalosporins could not be established, since various antimicrobial agents are con-
491 sidered as appropriate alternatives. However, the validity of this statement is limited by sever-
492 al aspects that need to be considered in the discussion of resistances of mastitis pathogens.

First of all, 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, whereas breakpoints for remaining β-lactams were not mastitis-specific. The Clinical and Laboratory Standards Institute (CLSI) published clinical breakpoints for 3 antimicrobial agents related to the indication mastitis: ceftiofur, penicillin / novobiocin and pirlimycin (Ruegg et al., 2015). These antimicrobial agents are either not approved for intramammary therapy or they 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)). Absence of specific clinical breakpoints for mastitis indication leads to a transfer of breakpoints established for different indications, other animal species or human medicine. The statement concerning the present resistance situation and especially recommendations for therapy resulting from such a classification are questionable. Haveri et al. (2005) also 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 rather on MIC values and distribution patterns of the isolates. The dosage achieved at the site of infection varies depending on the tissue and thus 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. A transmission of those breakpoints to mastitis pathogens is inappropriate and can lead to misinterpretations (Rossitto et al., 2002; Schwarz et al., 2010). For this reason, we emphasize the need to establish internationally standardiszed clinical breakpoints in order to adequately assess the resistance situation and derive effective recommendations for mastitis therapy.

CONCLUSION

In conclusion, the German *S. aureus* isolates investigated in the present study were inhibited at comparatively higher MIC_{90} values for most β-lactams than *S. aureus* isolates from Danish dairy farms. *BlaZ* and *mec*A resistance genes were isolated in individual German *S. aureus* isolates only and were generally associated with occurrence of comparatively higher MIC values. The predominant use of either penicillin in Denmark or cephalosporins of third (cefoperazone) and fourth (cefquinome) generation in Germany were not directly reflected in determined MIC values. Results further lead to the assumption 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 the results of the present study are not directly transferable to in vivo practice or derived therapy recommendations, thus highlighting the need for internationally standardized clinical breakpoints.

ACKNOWLEDGMENTS

We would like to thank the University of Copenhagen (Denmark) for supporting our study by providing Danish *S. aureus* isolates.

CONFLICT OF INTEREST

No conflict of interest.

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774 **Table 1**. Distribution of MIC of *S. aureus* (n = 85) isolated from 12 German dairy farms[1]

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

775

776 [1] MIC_{50} and MIC_{90} are displayed in italics and bold, respectively.

777 [2] Amoxicillin / clavulanic acid.

778 [3] Cefalexin / kanamycin.

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781

782 **Table 2.** Distribution of MIC of *S. aureus* (n = 93) isolated from 8 Danish dairy farms[1]

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

783

784 [1] MIC_{50} and MIC_{90} are displayed in italics and bold, respectively. Bold and italic digits indi-

785 cate that MIC_{50} and MIC_{90} were identical.

786 [2] Amoxicillin / clavulanic acid.

787 [3] Cefalexin / kanamycin.

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790

791 **Table 3**. MIC_{50} (µg/mL) and MIC_{90} (µg/mL) of German (n = 85) and Danish (n = 93) *S. aure-*

792 *us* isolates

Antimicrobial	MIC_{50} / MIC_{90} (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
Cefalexin / kanamycin	MIC_{50}	2	2
	MIC_{90}	4	2

793

794 **Table 4.** MIC values (µg/mL) of *S. aureus* isolates carrying *bla*Z and *mec*A (n = 5) and *bla*Z

795 (n = 1) from 3 German dairy farms

Resistance genes[1]	Dairy farm	MIC (µg/mL)							
		CEQ[2]	CEFO[3]	CPR[4]	PEN[5]	CLO[6]	OXA[7]	AMC[8]	CFX/K[9]
*bla*Z + / *mec*A +	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
*bla*Z + / *mec*A -	C	0.5	4	0.25	4	0.25	0.5	1	2

796

797 [1] "+" and "-" indicate a positive or negative result of resistance genes (*bla*Z, *mec*A), respec-

798 tively.

799 [2] Cefquinome.

800 [3] Cefoperazone.

801 [4] Cephapirin.

802 [5] Penicillin.

803 [6] Cloxacillin.

804 [7] Oxacillin.

805 [8] Amoxicillin / clavulanic acid.

806 [9] Cefalexin / kanamycin.

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808