Identification and characterization of methicillin-resistant coagulase-negative staphylococci from bovine mastitis

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Objectives: This study focused on the correlation between geno- and phenotypic tests in the correct assessment of mecA-mediated methicillin resistance among coagulase-negative staphylococci (CoNS) and the further characterization of mecA-positive isolates.

Methods: A total of 121 CoNS from cases of bovine mastitis were investigated for oxacillin susceptibility by disc diffusion and broth microdilution. Isolates classified as methicillin resistant by either method were tested by PCR for the mecA gene and the SCCmec type. The cefoxitin disc test was also applied. PFGE served to determine the genetic relationships of the resistant isolates.

Results: Sixteen isolates were classified as methicillin resistant and 96 isolates as methicillin susceptible by both methods. The mecA gene was identified in 15 of the 16 resistant isolates. Nine mecA-negative isolates showed oxacillin MICs of 0.5 or 1 mg/L, oxacillin zone sizes of 18–23 mm and were classified as methicillin susceptible in the cefoxitin disc test. SCCmec cassettes of types V (five Staphylococcus haemolyticus), III (one Staphylococcus saprophyticus), IV (five Staphylococcus epidermidis, one Staphylococcus capitis) and IV with an additional ccrA4/B4 gene (two S. epidermidis) were seen, while one S. epidermidis carried a non-typeable SCCmec element (mec complex B + no ccr gene complex detected). All isolates with SCCmec type IV or non-typeable cassettes exhibited low oxacillin MICs of 1–4 mg/L, whereas isolates with type III or V cassettes had MICs of ≥16 mg/L.

Conclusions: CoNS with oxacillin MICs of 0.5 and 1 mg/L should be confirmed for the presence of mecA before reporting them as methicillin resistant.

Keywords: breakpoints, oxacillin, SCCmec, molecular typing, Staphylococcus spp.

Introduction

Coagulase-negative staphylococci (CoNS) play a role as nosocomial pathogens in human medicine. They are often involved in foreign body infections and catheter-related infections, but also in urinary tract infections and endocarditis.1 In veterinary medicine, CoNS are mainly involved in inflammatory processes of the bovine udder. Mastitis represents the economically most important disease in the dairy industry worldwide. Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis and Escherichia coli are considered as the major pathogens in bovine mastitis.2 However, during recent years, CoNS have become the most common bovine mastitis isolates in many countries and are regarded as emerging mastitis pathogens.3 Antimicrobial agents are widely used in the treatment of udder infections. Among the antimicrobial agents approved for use in bovine mastitis, β-lactams, such as penicillins and cephalosporins, play a key role. Resistance to β-lactams in staphylococci is mediated by either β-lactamases of the BlaZ type or the mecA-encoded alternative penicillin-binding protein PBP2a, which shows a reduced binding to all β-lactam antibiotics currently available for mastitis therapy.2 Strains expressing the mecA gene are referred to as methicillin resistant. Since oxacillin maintains its activity during storage better than methicillin, laboratory diagnosis of methicillin resistance is based on the testing of oxacillin. According to recommendations of the CLSI, oxacillin-resistant Staphylococcus isolates shall be reported as resistant to other β-lactam antibiotics.3 Because of these implications for the choice of antimicrobial agents for therapeutic applications, it is of major importance.
to assess oxacillin resistance correctly. According to the CLSI document M31-A3, CoNS are classified as resistant by oxacillin MICs of $\geq 0.5$ mg/L and zone diameters of $\leq 17$ mm around a 1 $\mu$g oxacillin disc. These breakpoints, however, are not veterinary-specific breakpoints, but breakpoints adopted from human medicine. In addition, the CLSI also recommends a cefoxitin disc diffusion test for the prediction of mecA-mediated resistance in staphylococci. CoNS, for which zone diameters of $\leq 24$ mm around a 30 $\mu$g cefoxitin disc are recorded, shall be reported as oxacillin resistant. Again, this surrogate test is also adopted from human medicine and does not specifically refer to CoNS of animal origin.

To date, little is known about the correlation between geno- and phenotypic tests in the correct assessment of methicillin resistance among CoNS from animals. The aims of the present study were: (i) to comparatively screen CoNS from bovine mastitis for their oxacillin MICs and zone diameters; and (ii) to further analyse isolates that were classified as resistant by either test for their behaviour in the cefoxitin disc test, and for the presence of the mecA gene and the associated staphylococcal cassette chromosome mec (SCCmec) type.

Materials and methods

CoNS strains

A total of 121 CoNS isolates from individual cases of bovine mastitis were included in this study. These isolates were provided during 2003–09 from various diagnostic laboratories all over Germany on the default parameter of not more than one isolate per farm. Species identification was achieved by using the ID 32 STAPH system (bioMérieux, Nürtingen, Germany) supplemented by sequence analysis of part of the rpoB gene in eight cases where the ID 32 STAPH result was questionable or did not correspond to a profile stored in the ID 32 STAPH database. For rpoB amplification, the following primers were used: rpoB-fw (5'-CAATTCAATGGACCAAGC-3') and rpoB-rev (5'-CCGTCCCAAGTCAATGAA AC-3'). The PCR program included an initial denaturation at 94 $^\circ$C for 5 min, followed by 35 cycles of 94 $^\circ$C for 45 s, 52 $^\circ$C for 60 s, and 72 $^\circ$C for a final extension at 72 $^\circ$C for 10 min. The amplicons were sequenced and compared with the sequences deposited in the NCBI database (http://blast.ncbi.nlm.nih.gov/).

Oxacillin susceptibility testing

All 121 CoNS were tested for their oxacillin susceptibility by broth microdilution and disc diffusion, according to the recommendations given in the CLSI document M31-A3. For broth microdilution, custom-made microtitre plate panels (MCS Diagnostics, Swalmen, The Netherlands) were used. Scattergrams were constructed by plotting MICs against arithmetic averages, as previously described. The CLSI also recommends a cefoxitin disc diffusion test for the prediction of mecA-mediated resistance in staphylococci. CoNS, for which zone diameters of $\leq 24$ mm around a 30 $\mu$g cefoxitin disc are recorded, shall be reported as oxacillin resistant.

Detection of mecA and SCCmec typing

All CoNS identified by either phenotypic test as oxacillin resistant as well as the 13 isolates with an oxacillin MIC of 0.25 mg/L were screened by PCR for the presence of the mecA gene, as previously described. Isolates that were positive for the mecA gene were subjected to PCR-directed SCCmec typing. For this, multiplex 1 PCR, which detects mecA and the cassette recombinase complexes, as well as multiplex 2 PCR, which detects the mecA gene complex, were applied, as previously described. The ccrC amplicons were sequenced and compared with the ccrC sequences deposited in the NCBI database (http://blast.ncbi.nlm.nih.gov/).

PFGE

The mecA-positive CoNS were compared for their genetic relatedness by Smal PFGE using a previously described protocol. The Smal fragment patterns of the methicillin-resistant Staphylococcus haemolyticus isolates and the methicillin-resistant Staphylococcus epidermidis isolates were analysed with the GelCompar software package (Applied Maths, Kortrijk, Belgium), and the similarities between the profiles were calculated using the Dice coefficient with a maximum position tolerance of 1.2%. The patterns were clustered by using the unweighted pair group method with arithmetic averages, as previously described.

Results

Species identification and oxacillin resistance

A total of 15 different CoNS species were identified among the 121 isolates. The most predominant species were Staphylococcus chromogens (n=27) and Staphylococcus simulans (n=22), followed by Staphylococcus warneri (n=16), S. epidermidis (n=14), S. haemolyticus (n=13) and Staphylococcus xylosus (n=9). The remaining nine CoNS species (Staphylococcus saprophyticus, Staphylococcus hominis, Staphylococcus capitis, Staphylococcus hyicus, Staphylococcus sciuri, Staphylococcus arlettae, Staphylococcus cohnii ssp. urealyticus, Staphylococcus equorum and Staphylococcus gallinarum) were represented by one to five isolates. In two cases, the ID 32 STAPH profile suggested the presence of the coagulase-positive species Staphylococcus intermedius with probabilities of 83.4% or 99.0%, respectively. These isolates were coagulase-negative and rpoB sequencing identified them as S. chromogens. The six isolates that exhibited profiles that were not available in the ID 32 STAPH database proved upon rpoB sequencing to be S. chromogens (n=2), S. haemolyticus (n=2), S. warneri (n=1) and S. xylosus (n=1).

The susceptibility tests revealed that oxacillin MICs varied widely between 0.03 and 16 mg/L, with MIC50 and MIC90 values at 0.12 and 1 mg/L, respectively. The zone diameters around the 1 $\mu$g oxacillin disc varied for 110 isolates between 10 and 37 mm, while the remaining 11 isolates showed growth up to the disc. The scattergram that resulted from plotting MICs against zone diameters revealed that 16 isolates were classified as methicillin resistant and 96 isolates as methicillin susceptible by both methods (Figure 1). For this, the currently valid CLSI-approved breakpoints of oxacillin were applied. The mecA gene was identified in 15 of these 16 phenotypically resistant isolates. The single mecA-negative resistant isolate (S. arlettae) had an oxacillin MIC of 0.5 mg/L and a zone diameter of 16 mm, which classified this isolate as borderline resistant. However, the cefoxitin zone diameter of 30 mm identified this isolate as oxacillin susceptible. The 15 mecA-positive CoNS comprised 8 S. epidermidis, 5 S. haemolyticus, and single S. saprophyticus and S. capitis isolates. The S. capitis isolate had a cefoxitin zone diameter of 25 mm, which classified this isolate as borderline oxacillin susceptible. In contrast, all other
mecA-positive isolates had cefoxitin zone diameters of 12–23 mm, which classified them as oxacillin resistant. Another nine mecA-negative isolates (three S. saprophyticus, two S. sciuri, two S. xylosus, and single isolates of S. cohnii ssp. urealyticus and S. gallinarum) showed oxacillin MICs of 0.5 or 1 mg/L, had oxacillin zone sizes of 18–23 mm and were classified as oxacillin susceptible in the cefoxitin disc test by zone diameters of 26–32 mm. The 13 phenotypically susceptible isolates with an oxacillin MIC of 0.25 mg/L and oxacillin zone diameters of 23–29 mm were all mecA-negative.

Molecular analysis of mecA-positive CoNS isolates

SCCmec typing of the 15 mecA-positive CoNS isolates identified a SCCmec type V cassette (mec complex C+ccrC) in the 5 S. haemolyticus isolates. Sequence analysis of the ccrC amplicons identified ccrC2 and ccrC8 in single isolates, while a ccrC2 variant, which differed from ccrC2 by 3 bp, was seen in two S. haemolyticus isolates. One further S. haemolyticus isolate carried this ccrC2 variant in addition to ccrC8. A type III cassette (mec complex A+ccrA3/B3) was detected in the single S. saprophyticus, a type IV cassette (mec complex B+ccrA2/B2) in five S. epidermidis and the single S. capitis, and a type IV cassette with an additional ccrA4/B4 gene complex in two S. epidermidis. One S. epidermidis carried a SCCmec element non-typeable (mec complex B+no ccr gene complex detected) by the PCR approach used (Table 1). All isolates with SCCmec type IV and the non-typeable SCCmec element exhibited low oxacillin MICs of 1–4 mg/L, oxacillin zone diameters of 0–12 mm and cefoxitin zone diameters of 20–25 mm. In contrast, isolates with type III or type V SCCmec elements had distinctly higher oxacillin MICs of ≥16 mg/L, showed no zone of growth inhibition around the oxacillin disc and also had distinctly smaller cefoxitin zone diameters of 12–17 mm.

Although the CoNS isolates included in this study were from individual cases of mastitis, PFGE analysis was conducted to determine the genetic relationships of the mecA-positive S. haemolyticus (Figure 2a) and S. epidermidis isolates (Figure 2b). The single mecA-positive S. saprophyticus and S. capitis isolates were also subjected to PFGE analysis, but showed entirely different SmaI fragment patterns (data not shown). The five mecA-positive S. haemolyticus isolates

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**Figure 1.** Correlation between oxacillin zone diameters and oxacillin MICs for the 121 CoNS used in this study. The grey shaded area represents the ‘susceptible’ range whereas the black area indicates the ‘resistant’ range, based on the currently available breakpoints. The figures indicate the number(s) of CoNS that exhibit a certain zone diameter/MIC combination. The 15 mecA-positive, oxacillin-resistant CoNS are printed in bold type.
 originated from farms in two different regions in Germany (Baden-Württemberg and Bavaria) and showed individual fragment patterns, which exhibited similarities of 60%–85%. The eight mecA-positive S. epidermidis isolates originated from farms in three different regions (Rhineland-Palatinate, Bavaria and Brandenburg). Two isolates from Rhineland-Palatinate (RD 64 and RD 69) were indistinguishable by their SmaI fragment patterns. Six of the eight S. epidermidis isolates showed related patterns that clustered at 81% similarity, while the patterns of the remaining two isolates (3926 and coa 24) differed distinctly from one another and from the patterns of the aforementioned S. epidermidis isolates.

**Discussion**

During recent years, methicillin resistance in animals has gained particular attention from public health authorities. A recent review concentrated on meticillin-resistant *S. aureus* (MRSA) and *Staphylococcus pseudintermedius* (MRSP), both of which play a role as colonizers and pathogens in food-producing animals and companion animals, respectively. Colonized animals can pass meticillin-resistant staphylococci to humans who are in close contact with them. Infections in humans caused by livestock-associated MRSA—such as *S. pseudintermedius*—including MRSP—have been reported. In the case of MRSP, Bemis et al. demonstrated that both the CLSI breakpoints for oxacillin resistance and the cefoxitin disc test, as laid down in document M31-A3, classify a considerable number of mecA-positive *S. pseudintermedius* as oxacillin susceptible. The CLSI subcommittee on Veterinary Antimicrobial Susceptibility Testing carefully considered these data and recently adjusted the oxacillin breakpoints for *S. pseudintermedius* (http://data. memberclicks.com/site/aavld/Letter to the Editor.pdf). The MRSP example showed how important it is to have reliable breakpoints for the most accurate assessment of mecA-mediated resistance.

In the present study, nine mecA-negative CoNS (7.4%) were classified as oxacillin resistant by their MICs of 0.5 or 1 mg/L, but were classified as oxacillin susceptible by their oxacillin and cefoxitin zone diameters. Such isolates represent the ‘very major error’ group in MIC versus zone diameter scattergrams. In total, all six CoNS isolates with an oxacillin MIC of 0.5 mg/L and four of the seven CoNS isolates with an oxacillin MIC of 1 mg/L were mecA-negative, while all CoNS isolates with oxacillin MICs of ≥2 mg/L were mecA-positive (Figure 1). On the other hand, only a single isolate with a zone diameter of 16 mm was mecA-negative (Figure 1). This observation suggested that: (i) a MIC breakpoint of ≥0.5 mg/L for oxacillin resistance might also classify mecA-negative CoNS isolates as oxacillin resistant; and (ii) the currently available oxacillin zone diameters seemed to be more accurate indicators of mecA-mediated resistance in CoNS than oxacillin MICs. The CLSI document M100-S20, which includes interpretive criteria for the susceptibility testing of bacteria from humans, has already paid attention to similar observations made in human medicine and stated in the comments box of Table 2c for *Staphylococcus* spp. that ‘Oxacillin interpretive criteria may overcall resistance for some coagulase-negative staphylococci because some non-*S. epidermidis* strains for which the oxacillin MICs are 0.5 to 2 mg/L lack mecA. For serious infections with coagulase-negative staphylococci other than *S. epidermidis*, testing for mecA or for PBP2a or with cefoxitin disc diffusion may be appropriate for strains for which oxacillin MICs are 0.5–2 mg/L.’ Based on the observations made in this study, a similar recommendation to test CoNS with oxacillin MICs of 0.5 and 1 mg/L for the presence of mecA or for PBP2a before reporting them as meticillin resistant should be included in the respective comments box of the forthcoming CLSI document M31-A4. These additional tests may

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<th>Isolate ID</th>
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<th>ccr gene complex</th>
<th>ccr complex</th>
<th>SCCmec type</th>
<th>Oxacillin MIC (mg/L)</th>
<th>Oxacillin zone diameter (mm)(^a)</th>
<th>Cefoxitin zone diameter (mm)(^b)</th>
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\(^a\)1 μg oxacillin disc.  
\(^b\)30 μg cefoxitin disc.  
\(^c\)ccrC2-v=ccrC2 variant described in the text.
prevent potentially efficacious cephalosporins from being excluded from mastitis therapy if the phenotypically oxacillin-resistant CoNS are mecA-negative.

While the molecular characteristics of methicillin-resistant S. aureus from bovine mastitis have been published,17–20 little information is available on methicillin-resistant CoNS (MRCoNS) from dairy cattle.21–23 In the present study, S. epidermidis and S. haemolyticus represented the most frequently observed species among the MRCoNS. A study from Mexico identified these two species also as the most predominant species among MRCoNS from infections of humans.24 Similar results were obtained from human patients in Algeria, Mali, Moldavia and Cambodia, in which S. epidermidis and S. haemolyticus comprised 98% of the 96 MRCoNS tested.25 SCCmec typing identified mainly type IV elements among S. epidermidis isolates from humans in various parts of the world.24–27 A single S. epidermidis isolate of animal origin was also found to harbour a type IV SCCmec element.28 One of the methicillin-resistant S. epidermidis of the present study harboured a ccrC2 variant in addition to ccrC8. The combination of ccrC2 and ccrC8 has so far only been described in a variant of an SCCmec type V element of S. aureus.29,30 In accordance with the findings of the present study, methicillin-resistant S. haemolyticus from humans also carried mainly SCCmec elements of type V.25,31 The predominant occurrence of a specific SCCmec type in a certain CoNS species should be considered with discretion. Studies on larger test populations showed that besides the dominant SCCmec type, various other types may occur in members of the same CoNS species.25,26

Species identification of CoNS from animals is a cumbersome process. A recent study on species identification of CoNS from bovine milk samples showed that the identification of certain species, such as S. chromogenes, S. haemolyticus and S. warneri, via ID 32 STAPH is particularly problematic.32 The results of the present study confirmed these findings, with seven of the eight misidentified or unidentified CoNS isolates belonging to these three species.

In conclusion, the results of this study confirmed that the currently available oxacillin breakpoints are not able to correctly differentiate between mecA-positive and mecA-negative CoNS isolates. The application of additional tests, such as mecA PCR, may help to reliably detect the presence of the mecA gene in
questionable isolates. Further molecular studies of mecA-positive bovine S. epidermidis and S. haemolyticus showed parallels to the SCCmec types seen among corresponding MRCoNS from human infections.

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None to declare.

References

Berglund C, Söderquist B. The origin of a methicillin-resistant *Staphylococcus aureus* isolate at a neonatal ward in Sweden—possible horizontal transfer of a staphylococcal cassette chromosome mec between methicillin-resistant *Staphylococcus haemolyticus* and *Staphylococcus aureus*. *Clin Microbiol Infect* **2008; 14**: 1048–56.