Characterization of methicillin-resistant non-Staphylococcus aureus staphylococci carriage isolates from different bovine populations

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Objectives: This study aimed at investigating bovine non-S. aureus staphylococci for their role as a potential reservoir for methicillin resistance.

Methods: Nasal swab samples were collected from 150 veal calves on 15 veal farms, 100 dairy cows on 10 dairy farms and 100 beef cows on 10 beef farms. Suspected staphylococcal isolates were investigated by PCR for the presence of the classic mecA and mecA_LGA251. Methicillin-resistant non-S. aureus staphylococci (MRNAS) were genotypically identified and were characterized by broth microdilution antimicrobial susceptibility testing and staphylococcal cassette chromosome mec (SCCmec) typing.

Results: The MRNAS (n=101) carriage rate was estimated as 30.29% (95% CI 6.14%–74.28%) in veal calves, 13.1% (95% CI 1.28%–63.72%) in dairy cows and 24.8% (95% CI 11.97%–44.42%) in beef cows. Carriage rates were not significantly different between the three populations (P>0.05). mecA_LGA251 was not detected. Most (n=80) MRNAS were identified as Staphylococcus sciuri, Staphylococcus lentus or Staphylococcus fleurettii. Resistance to aminoglycosides, macrolide–lincosamide–streptogramin antimicrobials, tetracycline and ciprofloxacin was frequently detected. Two linezolid-resistant MRNAS from veal calves carried the multidrug-resistance gene cfr. SCCmec cassettes of type III predominated (n=46); another 40 SCCmec cassettes harboured a class A mec complex without identifiable ccr complex; type IVa, type V and several other non-typeable cassettes were detected in low frequencies, especially in methicillin-resistant Staphylococcus epidermidis.

Conclusions: The SCCmec types predominating in bovine MRNAS differ from those mostly detected in livestock-associated methicillin-resistant S. aureus strains. Yet, the detection of cfr and the high level of other antimicrobial resistances suggest a potentially important role of bovine MRNAS as a reservoir for resistance determinants other than SCCmec.

Keywords: coagulase-negative staphylococci, cattle, Staphylococcus sciuri, SCCmec, cfr

Introduction

Methicillin-resistant staphylococci have long been known as important human pathogens.1 The mecA gene, which mediates methicillin resistance, is located on a mobile genetic element called staphylococcal cassette chromosome mec (SCCmec), which integrates into the staphylococcal chromosome.2 Depending on structural composition, so far, 11 different SCCmec types (I–XI) and numerous subtypes have been recognized in methicillin-resistant Staphylococcus aureus (MRSA).3–6 However, the SCCmec diversity extends beyond these (sub)types, especially in methicillin-resistant non-S. aureus staphylococci (MRNAS).3,7 Therefore, MRNAS have been proposed to function as an SCCmec reservoir for S. aureus.7 Indirectly, transfer of (parts of) SCCmec between MRNAS and S. aureus has been shown in several studies.8–10

In veterinary medicine, increased attention has been drawn to the manifestation of methicillin-resistant staphylococci after the unexpected detection of a particular type of MRSA, sequence type (ST) 398, in pigs.11,12 Since, MRSA ST398 has been found to be present in pigs worldwide and to affect various other animal species, including bovines.13–15 In contrast, so far, the carriage
and diversity of MRNAS in food-production animals and their potential as an SCCmec reservoir has been poorly assessed. While some recent studies characterized the MRNAS reservoir in pigs, less is known on bovines. Recently, a variant of the classic mecA gene, designated mecALGA251, and carried in a deviant SCCmec type XI, was discovered in MRSA from dairy cows (DCs) and other species in several European countries. Similar as suggested for classic mecA, MRNAS could function as the evolutionary source and/or reservoir of mecALGA251. Thus far the presence of this gene has not been investigated in MRNAS from bovines.

The present study aimed at investigating the carriage and species diversity of MRNAS, harbouring either classic mecA or mecALGA251, in different bovine populations (veal calves (VCs), DCs and beef cows (BCs)). Moreover, the role of bovine MRNAS as reservoirs for methicillin and other antimicrobial resistances was assessed through SCCmec typing and phenotypical determination of additional antimicrobial resistances.

**Methods**

**Sampling**

Nasal swab samples were collected from 150 VCs, 100 DCs and 100 BCs. VCs originated from 15 Belgian veal farms exclusively breeding VCs, while DCs and BCs originated each from 10 Belgian farms exclusively breeding DCs and BCs, respectively. All farms were randomly selected from the national database of the Belgian Federal Agency for the Safety of the Food Chain. Ethics approval was not required for this study under Belgian regulations, as taking a nasal swab does not cause pain, distress or lasting harm.

On each farm, a convenience sample of 10 animals, evenly distributed over the farm, was selected. All animals were healthy at the moment of sampling (based on visual inspection and information from the farmer). VCs were between 3 and 30 weeks of age; apart from seven heifers, all DCs were at least 2 years of age; all BCs were at least 2 years of age. Samples were taken and stored as previously described.

**Bacterial isolates**

Isolation, selection and storage of isolates was done as previously described. As stated there, the term ‘isolate’ further refers to a pure culture showing a colony morphotype unique for a given sample.

**Detection of mecA, mecALGA251 and identification of MRNAS**

Detection of mecA in non-S. aureus staphylococci (NAS) was done as previously described. MRSA strain NCTC10442, methicillin-susceptible S. aureus strain ATCC 25923, Escherichia coli strain ATCC 25922 and a methicillin-resistant Staphylococcus haemolyticus field strain were used as control strains. All NAS isolates negative for mecA were also investigated for mecALGA251 using previously described primers: mecALGA251-MultIP (GAAA AAAAGCTTGAAACGGCTC), mecALGA251MultIP (GAAGATCTTGGCCCCGTGC) and mecALGA251-IP (CCTGAATCTWGCTTAATAATTTGC). MRSA strain NCTC 10442 and MRSA field isolates found positive for mecALGA251 were included as negative and positive control strains, respectively. All NAS positive for mecA or mecALGA251 were further identified to the species level as previously described.

**Antimicrobial susceptibility testing**

For all NAS positive for mecA or mecALGA251, MICs of 19 antimicrobials (penicillin, cefoxitin, kanamycin, streptomycin, gentamicin, erythromycin, clindamycin, quinupristin/dalfopristin, linezolid, tiamulin, chloramphenicol, rifampicin, ciprofloxacin, fusidic acid, tetracycline, trimethoprim, sulfamethoxazole, vancomycin and mupirocin) were determined using custom veterinary international Sensititre staphylococci plates EUST (Trek Diagnostics System, UK), according to the manufacturer’s instructions. CLSI susceptibility breakpoints were used for qualitative interpretation of MIC values (with isolates showing an ‘intermediate’ MIC being counted as resistant). Refer to Table 1 and Table S2, available as Supplementary data at JAC Online for the tested MIC ranges and for further explanation on the use of the susceptibility breakpoints. For vancomycin and mupirocin, which are not included in these tables, the MIC ranges tested were 1–16 mg/L and 0.5–2 mg/L + 256 mg/L, respectively. The CLSI susceptibility breakpoint for vancomycin was ≤2 mg/L. The susceptibility breakpoint used for mupirocin was ≤4 mg/L, based on the literature. MRNAS isolates that appeared resistant to linezolid (MIC >4 mg/L) were tested for the presence of the cfr gene.

**Testing for PBP2a production**

All isolates showing discordant results for mecA PCR and cefoxitin MICs, i.e. mecA positive and MICs ≤4 mg/L, were tested for the production of PBP2a as previously described.

**Typing of SCCmec elements**

The SCCmec type of all MRNAS was determined as previously described.

**Statistical analysis**

MRNAS carriage rates in the total bovine population and in the separate populations (VCs, DCs and BCs) were estimated using a logistic regression analysis that took into account both the sampling probability of each animal and the herd clustering effect. The type of production was considered as a categorical explanation variable and coded accordingly. The P values associated with the specific group coefficient were checked for significance (P<0.05) with the reference group (calves) then were run again considering each other group as the reference.

**Results**

**Detection and identification of MRNAS**

The presence of mecA or mecALGA251 was set as the gold standard to consider an isolate as an MRNAS. In total, the mecA gene was detected in 101 isolates, originating from 82 of the 350 sampled animals, corresponding to an estimated MRNAS carriage rate of 21.4% (95% CI 12.1%–34.9%). The mecALGA251 gene could not be detected in any of the isolates.

In the three bovine populations, carriage rates of 30.29% (95% CI 6.14%–74.28%) for VCs, 13.10% (95% CI 1.28%–63.72%) for DCs and 24.8% (95% CI 11.97%–44.42%) for BCs were estimated (Table S1, available as Supplementary data at JAC Online). These carriage rates were not significantly different from one another (P > 0.05).

Overall, seven MRNAS species were found (Table S1, available as Supplementary data at JAC Online). MRNAS belonging to the *Staphylococcus sciuri* species group (consisting of *S. sciuri*, *Staphylococcus lentus*, *Staphylococcus fleurettii* and *Staphylococcus*
Table 1. MICs for MRNAS species from bovines [S. epidermidis \( n = 13 \), S. haemolyticus \( n = 5 \), S. lentus \( n = 24 \), S. sciuri \( n = 33 \) and S. fleurettii \( n = 23 \)] of 11 antimicrobial agents

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Antimicrobial susceptibility testing and PBP2a testing

All MRNAS isolates were susceptible to vancomycin (86.1% of isolates with MIC ≤1 mg/L; 13.9% with MIC = 2 mg/L) and mupirocin (60.4% of isolates with MIC ≤0.5 mg/L; 12.9% with MIC = 1 mg/L; 26.7% with MIC = 2 mg/L).

The MIC distributions for the β-lactam antimicrobials penicillin and cefoxitin are shown in Table S2 (available as Supplementary data at JAC Online). As for fusidic acid, rifampicin, streptomycin and tiamulin, no CLSI susceptibility breakpoints are available, no qualitative interpretation was done for these four antimicrobials, and the MIC distributions for these agents are also shown in Table S2, available as Supplementary data at JAC Online. The MIC distributions for the remaining 11 antimicrobial agents are shown in Table 1.

Among all species, isolates were found that were phenotypically susceptible to the β-lactam antimicrobials penicillin (total n = 14) and cefoxitin (total n = 46). Most notable were the results for MR S. lentus, of which 54.1% of the isolates had cefoxitin MICs ≤4 mg/L, and MR S. fleurettii, of which 100% of the isolates had cefoxitin MICs ≤4 mg/L. Nonetheless, 43 of the 46 cefoxitin-susceptible isolates were found to produce PBP2a. The three non-PBP2a producers were two MR S. fleurettii from BCs and the single MR Staphylococcus arlettae isolate; all three had cefoxitin MICs of 4 mg/L.

Single isolates of MR S. sciuri and MR S. lentus, both from VCs, appeared resistant to linezolid (MICs = 8 mg/L) (Table 1). These isolates displayed the PhLOPSA phenotype (resistance to penicillins, lincosamides, oxazolidinones, pleuromutilins and streptogramin A antimicrobials) and were positive for the cfr gene.

Both MR Staphylococcus equorum isolates were resistant to streptomycin, erythromycin and tetracycline. In addition, the isolate originating from VCs showed resistance to kanamycin, chloramphenicol, clindamycin and trimethoprim. The single MR S. arlettae isolate was resistant to clindamycin and quinupristin/dalfopristin.

Typing of SCCmec elements

Eleven different ccr-mec-complex combinations were detected in the 101 MRNAS, defining three recognized SCCmec types, three variants of SCCmec types and five non-typeable (NT) variants (Table 2). Recognized SCCmec types and their variants made up slightly more than half (54.5%) of all SCCmec elements, with 45.5% cassettes of type III, 5.9% cassettes of type V and 3.0% cassettes of type IV. The most prevalent NT variant was by far NT 3, representing 80% of NT cassettes and 39.6% of all SCCmec elements. Two NT 5 elements, for which PCR products for neither the ccr gene complex nor the mec gene complex could be amplified, were detected in the two non-PBP2a-producing S. fleurettii isolates; the third was found in an MR S. haemolyticus isolate with cefoxitin MIC >16 mg/L.

The largest diversity of SCCmec elements was observed in MRNAS from VCs, including all 11 above-mentioned SCCmec types or variants (Table 2). The predominant SCCmec cassettes...
in VCs were type III (3A), being present in 53.4% of isolates. When including types IIIvar1 and IIIvar2, 72.4% of MRNAS from VCs harboured a type III SCCmec element. In DCs and BCs, NT 3 SCCmec elements predominated (84.6% of DC isolates and 80% of BC isolates).

When regarding the different MRNAS species, MR S. sciuri and MR S. lentus carried mainly type III or IIIvar2 SCCmec elements and less frequently NT 3 (Table 2). NT 3 cassettes were detected in five MRNAS species, but were mainly associated with MRNAS from the S. sciuri species group, and specifically MR S. fleurettii isolates. MR S. epidermidis harboured the largest diversity of SCCmec elements, with a total of nine different SCCmec types and six types that were exclusively detected in MR S. epidermidis. The non-PBP2a-producing MR S. arlettae isolate possessed a type III (3A) SCCmec element.

**Discussion**

To our knowledge, this is the first study on MRNAS in bovines that distinguishes between the three main cattle breeding practices that are executed worldwide. It must be noted that the housing of the animals is an important aspect differing between the three populations. In Belgium, DCs and BCs are housed all together or in relatively big groups and therefore they are to be regarded as in-contact animals. VCs on the other hand are kept individually until the age of 8 weeks and later on in small groups—from which always only one animal was sampled. Therefore, the animals sampled on a given veal farm can be considered independent with respect to direct contact. The difference in clustering of the animals has been accounted for in the statistical analysis of the carriage rates. However, various other factors may have an effect on MRNAS carriage and the extent to which they might have influenced our data is not clear.

The total MRNAS carriage rate that was estimated is much lower than the 61% reported for Swiss bovines. Yet, the carriage rate observed in our VCs was comparable to the 36.6% found in France.

Few literature is available on the species diversity of NAS residing in the nares of cattle, rendering it difficult to assess the diversity of our MRNAS collection. Yet, compared with the results from recent studies in pigs, we found a rather low MRNAS species diversity. The observed predominance of the S. sciuri species group in MRNAS has previously been reported in bovines and has also been described in pigs, chickens, goats and sheep, and horses, highlighting the role of the S. sciuri species group as the most widespread MRNAS species among many animal species.

Although our MRNAS isolates were selected based on the presence of mecA, several isolates appeared phenotypically susceptible. As production of PBP2a was shown for most of the cefoxitin-susceptible isolates, proving the functionality of mecA and its successful transcription and translation, our data illustrate that the cefoxitin MIC is a poor indicator for mecA-mediated methicillin resistance in non-S. aureus staphylococci from animals.

Considering the large number of antimicrobial classes to which resistance is frequently observed (Table 1), our data suggest that MRNAS from bovines potentially function as an important reservoir for various antimicrobial resistance determinants. Future research will establish the presence of resistance...
genes among the MRNAS and investigate the similarity with genes carried by bovine MRSA strains.

Resistance rates differed among the MRNAS species, being generally higher in MR S. epidermidis, MR S. haemolyticus and MR S. lentus compared with MR S. sciuri and MR S. fleurettii. Interestingly, the former species were largely associated with VCs, while MR S. fleurettii was mainly found in BCs. Moreover, among MR S. sciuri, additional antimicrobial resistances were seen more frequently in the isolates from VCs than those from DCs and BCs. Indeed, in this species, the medians of the frequency distribution of the resistance to antimicrobials for VCs, DCs and BCs were 5, 2 and 3, respectively (data matrix not shown). This might indicate that the species-related difference is in fact animal related and hence that aspects related to the age of the animals or to management practices might explain the different antimicrobial resistance rates. Indeed, onveal farms, upon arrival of calves on a farm, several group antimicrobial therapy courses are typically applied, while in dairy and beef farms, antimicrobial use is more restricted and applied individually.31 However, it is important to note that only MRNAS were studied, which are probably not representative for the general non-S. aureus staphylococcal population residing in the nares of the different bovine populations.

As linezolid is not used in veterinary medicine, detecting two linezolid-resistant MRNAS from VCs was very remarkable. There- plasticity of SCC mec has also been described in other studies.18,27 This might indicate that the species-related difference is in fact animal related and hence that aspects related to the age of the animals or to management practices might explain the different antimicrobial resistance rates. Indeed, onveal farms, upon arrival of calves on a farm, several group antimicrobial therapy courses are typically applied, while in dairy and beef farms, antimicrobial use is more restricted and applied individually.31 However, it is important to note that only MRNAS were studied, which are probably not representative for the general non-S. aureus staphylococcal population residing in the nares of the different bovine populations.

As linezolid is not used in veterinary medicine, detecting two linezolid-resistant MRNAS from VCs was very remarkable. Therefore, we speculated that this resistance phenotype would have been caused by the presence of a gene conferring cross-resistance, and the presence of the cfr gene was proven.25 This gene was initially identified in a S. sciuri isolate obtained from the nasal swab of a bovine calf,23,32 so the current detection of cfr in two MRNAS from VCs might not be surprising. Nonetheless, it is a worrisome observation, as the gene is usually plasmid borne13,32,33 and its detection seems to be increasing, having recently been detected in porcine MRSA ST398 and bovine MRSA ST9 isolates3,17,36 and appearing to be capable of spreading among various other animal-associated bacteria.37–39 cfr has also been identified in linezolid-resistant staphylococci causing human infections,40,41 including outbreaks.42,43 This illustrates the mobility of cfr and warns us to actively monitor its reservoir in (animal) staphylococci.

The SCCmec typing results were quite different compared with those observed in MRNAS from Belgian pigs. In the latter, recognized SCCmec types constituted 80% of all SCCmec elements and type IV SCCmec elements were largely predominant.17 Currently, recognized SCCmec types formed a minority and cassettes of type III were most common. This difference is to be explained by the apparent association between type III cassettes and MRNAS species of the S. sciuri species group, which has also been described in other studies.18,27

It was remarkable to find nine different SCCmec elements in barely 11 MR S. epidermidis isolates. This might indicate a high plasticity of SCCmec in bovine MR S. epidermidis, as was previously shown for human MR S. epidermidis strains.44 Not solely in humans, but equally in animals, MR S. epidermidis might act as a continuous source for new SCCmec variants that might then be transferred to S. aureus or other NAS.44

The predominance of NT 3 among the NT SCCmec elements must be interpreted with caution. NT 3 elements shared the presence of a class A mec gene complex and the inability to detect ccr genes, so it is fairly possible that the ccr complexes among the NT 3 carriers varied or were even absent. Indeed, while our PCR method is only able to detect ccrA/B allotypes 1–4 and ccrC1, various other allotypes have been described in recent years.3,4,5,45–47 The predominance of NT 3 elements in S. fleurettii requires special attention. Recently, a S. fleurettii mecA gene and class A mec gene complex-like region have been described.20 These structures were assumed to be native to S. fleurettii due to the association with genes essential for cell growth instead of with SCCmec structures. Hence, it is possible that the NT 3 ‘cassette’ we found in our S. fleurettii isolates reflects these native structures. Future research is needed to elucidate this.

This study aimed to assess the potential of bovine MRNAS to serve as a reservoir for SCCmec, particularly in relation to livestock-associated MRSA, and especially MRSA CC398. Our results suggest that this potential is quite low. Indeed, some elements of type IVa and V, which are typical for MRSA CC398, were found, especially in VCs. Yet, there was generally little agreement in the SCCmec types that dominate in bovine MRNAS and MRSA CC398. This might partially explain why, so far, MRSA CC398 has not been described more often in DCs and BCs, except for some reports on MRSA CC398 in mastitis.13,15,48,49 Yet, in VCs, MRSA CC398 is more frequently detected16,18,48 and carriage rates of MRSA CC398 equal those in pigs.14 Still, also there, MRNAS possessing SCCmec types IVa or V were present in low frequencies. This indicates that other factors than solely the presence of an SCCmec reservoir play a role in the emergence or maintenance of MRSA CC398 on a farm.

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We are grateful to Katrien Geurts and Deborah Pétrone for excellent technical assistance. Maria de los Angeles Argudín is acknowledged for her critical review of the manuscript prior to submission.

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Transparency declarations
None to declare.

Supplementary data
Tables S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


