Characterization of methicillin-resistant Staphylococcus sciuri isolates from industrially raised pigs, cattle and broiler chickens

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Objectives: This study aimed at assessing the epidemiology and genetic diversity of methicillin-resistant Staphylococcus sciuri (MRSS) from different farm animal species.

Methods: Nasal swabs were collected from 200 pigs, 100 dairy cows, 100 beef cows, 150 veal calves and 200 broilers. Colonies were isolated on selective media containing cefoxitin and the mecA gene was detected by PCR. Antimicrobial resistance was determined by broth microdilution. The genetic diversity was assessed by PFGE and resistance and virulence genes were detected by microarray analysis.

Results: The total MRSS prevalence at the animal level was estimated at 9.5%, varying from ≈10% in veal (13.3%), broilers (12.5%) and dairy cows (10.0%) to 6.5% in pigs and 3.0% in beef cows. mecA was detected in all isolates. SCCmec elements of type III and non-typeable ones were seen most frequently. More than 90% of isolates were non-wild-type (NWT) for gentamicin, penicillin, tiamulin, clindamycin and quinupristin/dalfopristin. The frequency of NWT isolates for fusidic acid and trimethoprim ranged between 78% and 87%. PFGE analysis allowed distinction between two major clusters. Most isolates tested by microarray carried erm and tet genes. Virulence genes were also detected, including an isa gene encoding an immune-evasion factor and the hsdS2 gene encoding a site-specific deoxyribonuclease.

Conclusions: This study shows that multiresistant MRSS is carried by different farm animal species. Although some animals shared the same strain, PFGE showed different patterns, indicating high diversity among the MRSS isolates recovered. The absence of clusters associated with a certain animal species suggests low host specificity.

Keywords: coagulase-negative staphylococci, livestock, S. sciuri, SCCmec, antimicrobial resistance, PFGE, microarrays

Introduction

Methicillin resistance in staphylococci, referring to resistance to all β-lactam antimicrobials, is an important concern in both human and veterinary medicine.1 The most important gene encoding methicillin resistance is the mecA gene, which is located on a mobile genetic element called staphylococcal cassette chromosome mec (SCCmec).2 The mecA gene is widespread in Staphylococcus aureus and a variety of coagulase-negative staphylococci (CoNS) species of both human and animal origin.3,4 In 2011, an unknown variant of mecA, called mecC, was described in human and bovine populations in the UK.5 Members of the Staphylococcus sciuri species group also include S. sciuri with three subspecies, Staphylococcus lentus, Staphylococcus vitulinus, Staphylococcus fleurettii and Staphylococcus stepanovicii, are of special importance in relation to the origin and evolution of methicillin resistance genes. Indeed, S. fleurettii has been shown to carry the putative evolutionary ancestor of mecA.6 Furthermore, other mecA homologues appear to occur naturally in S. sciuri and S. lentus.7 Since these homologues do not always confer phenotypic resistance to methicillin,8 S. sciuri are often considered as methicillin susceptible.9 However, several studies showed methicillin resistance in S. sciuri.10,11

S. sciuri has long been considered as a non-pathogenic commensal bacterium, mostly recovered from skin and mucous membranes of warm-blooded animals.12,13 However, it is...
now also recognized as a potential pathogen responsible for various diseases such as mastitis, dermatitis and epidermitis in animals. Meticillin-resistant CoNS (MRCoNS) have been proposed to be a potential reservoir of SCCmec elements for S. aureus. Furthermore, resistance genes and pathogenicity markers of S. aureus have been recovered in meticillin-resistant S. sciuri (MRSS) isolates from chickens. It is, though, important to investigate this in a more global context in order to have a general overview of the role of MRSS as a potential reservoir of resistance and virulence genes for meticillin-resistant S. aureus (MRSA).

The present study aimed at estimating the genetic diversity of MRSS isolated from healthy farm animals by means of SCCmec typing and PFGE. Moreover, the role of MRSS as a potential resistance and virulence gene reservoir for other staphylococci was assessed through antimicrobial susceptibility testing and the detection of antimicrobial resistance and virulence genes.

Materials and methods

Sampling and isolation methods

Nasal swab samples were collected in Belgium from 10 pig farms, 10 dairy farms, 10 beef farms, 15 veal farms and 10 broiler farms. A total of 200 pigs, 100 dairy cows, 100 beef cows, 150 veal calves and 200 broilers were sampled. The samples collected from pigs and bovines have been described previously. The samples were enriched in brain heart infusion (bioMérieux, France) and 3.5 mg/L cefoxitin (Sigma–Aldrich, USA), on Columbia agar (Oxoid, Germany) supplemented with 5% sheep blood (bioMérieux, France) and 3.5 mg/L cefoxitin (Sigma–Aldrich, USA), on ChromID S. aureus agar (bioMérieux, France) and on Columbia colistin/aztreonam agar supplemented with 5% sheep blood (Oxoid, France) as described previously. Colonies were then purified on blood agar plates and visually inspected after 48–72 h at 37°C in order to improve pheno- typic identification.

Identification, mecA detection and SCCmec typing

DNA was extracted as previously described. Detection of the mecA and mecC genes, identification and SCCmec typing were performed as described previously. The SCCmec typing results for broilers are original data while those for pigs and bovines refer to previously published studies.

Macrorestriction PFGE analysis

Whole genome DNA of isolates was prepared and digested by the SmaI restriction enzyme before performing PFGE using a CHEF Mapper XA System (Bio-Rad Laboratories, UK). Plugs were prepared according to the protocol of Chung et al. with modifications. Plugs were prepared using Bio-Rad CHEF System plug moulds and subjected to restriction with SmaI (Fermentas, Belgium) following the manufacturer’s instructions. The electrophoresis conditions were 6 V/cm in 0.5x TBE buffer (45 mM Tris/45 mM boric acid/1 mM EDTA (pH 8)) at 11.3°C and runs lasted 23 h with switch times from 5 to 35 s. PFGE profiles were compared using BioNumerics software (Version 6.6, Applied Maths, Belgium). A dendrogram was derived from Dice similarity indices based on the unweighted pair group method with arithmetic averages. S. aureus NCTC 8325 (National Collection of Type Cultures, UK) was included as the size standard for PFGE analysis.

Antimicrobial susceptibility testing

Antimicrobial resistance was determined using broth microdilution (Sensititre, Trek Diagnostic Systems, OH, USA) following the manufacturer’s instructions. Susceptibility to 19 antibiotics (penicillin, cefoxitin, kanamycin, streptomycin, gentamicin, erythromycin, clindamycin, quinupristin/dalfopristin, linezolid, tiamulin, chloramphenicol, rifampicin, ciprofloxacin, fusidic acid, tetracycline, trimethoprim, sulfamethoxazole, vancomycin and mupirocin) was tested. The concentrations tested are shown in Table 2. The results corresponding to the bovine isolates were published previously. The MICs were interpreted using EUCAST epidemiological cut-offs (ECOFFs) for CoNS. If no ECOFF was available, then the ECOFF for S. aureus was used. Data from the EUCAST MIC distribution web site were last accessed on 16 January 2014 (http://www.eucast.org). When no ECOFF for CoNS or S. aureus was available, determination of wild-type (WT) and non-WT (NWT) was based on the frequency distribution of the MICs as previously described.

DNA microarray-based typing and detection of resistance and virulence genes

Twenty-two isolates were selected based on the different antimicrobial resistance phenotypes and PFGE profiles, in order to obtain the largest diversity. Microarray analysis was performed on these strains using the Identibac S. aureus Genotyping DNA Microarray (Alere Technologies, Jena, Germany) according to the manufacturer’s instructions. The DNA microarray covers 333 oligonucleotide probes, detecting resistance and virulence genes. A full list including primer and probe sequences is available online (http://alere-technologies.com/en/products/lab-solutions/s-aureus.html).

Results

MRSS prevalence at animal level

Seventy-one (9.5%, 95% CI 7.4–11.6) MRSS were identified. Thirteen (6.5%, 95% CI 3.1–9.9) of these isolates originated from pigs, 25 (12.5%, 95% CI 7.9–17.1) from broilers and 33 (9.4%, 95% CI 6.4–12.5) were isolated from bovines. Among these, 20 (13.3%, 95% CI 7.9–18.8) originated from veal calves, 10 (10.0%, 95% CI 4.1–15.9) from dairy cows and 3 (3.0%, 95% CI 0.0–6.3) from beef cows (Table 1).

SCCmec and macrorestriction PFGE analysis

Most isolates possessed the SCCmec type III, of which 45 (63.4%) were type III and 10 (14.1%) were type IIIA (Table 1). The remaining 16 isolates (22.5%) showed only the mecA gene but no ccr complex was detected with the PCR. These were thus considered non-typeable. However, among the five isolates carrying a non-typeable SCCmec that were tested with the microarray, two were positive for ccrB-1 or for ccrA-3 and ccrB-3. Isolates from broilers carried mostly SCCmec type III and type IIIA with 23 (92.0%) and 2 (8.0%) isolates, respectively. Among the 71 MRSS isolates studied, 5 isolates did not show interpretable profiles in PFGE analysis and were thus considered non-typeable. PFGE analyses showed 37 different profiles grouped in two major clusters at 40% similarity (Figure 1). The first cluster was composed of 25 isolates grouped in 14 profiles. This cluster was composed of nine (36.0%) isolates from veal calves, eight (32.0%) from broilers, five (20.0%) from pigs and three (12.0%) from beef cows. This cluster comprised also 15 (60.0%) SCCmec
type III, seven (28.0%) type IIIA and three (12.0%) non-typeable SCCmec. The second cluster was composed of 41 isolates grouped in 23 profiles. This cluster was composed of 16 (39.0%) isolates from broilers, nine (22.0%) from dairy cows, eight (19.5%) from pigs and eight (19.5%) from veal calves. This cluster comprised also 24 (58.5%) SCCmec type III, 15 (36.6%) non-typeable SCCmec and 2 (4.9%) SCCmec type IIIA.

Antimicrobial resistance
All isolates were NWT for gentamicin. Most isolates were NWT for penicillin (98.6%, 95% CI 98.3–98.9), clindamycin (98.6%, 95% CI 98.3–98.9), tiamulin (98.6%, 95% CI 98.3–98.9), quinupristin/dalfopristin (91.5%, 95% CI 90.8–92.3), fusidic acid (87.3%, 95% CI 86.4–88.2) and trimethoprim (78.9%, 95% CI 77.7–80.0). More than half of the isolates were NWT for tetracycline (62.0%, 95% CI 60.6–63.3), mupirocin (56.3%, 95% CI 55.0–57.7) and erythromycin (52.1%, 95% CI 50.7–53.5). A little more than 25% of the isolates were additionally NWT for streptomycin (28.2%, 95% CI 26.9–29.4) and ~10% for kanamycin (14.1%, 95% CI 13.1–15.0) and ciprofloxacin (9.9%, 95% CI 9.0–10.7). Few isolates belonged to the NWT population for chloramphenicol (8.5%, 95% CI 7.7–9.2), sulfamethoxazole (4.2%, 95% CI 3.7–4.8), linezolid (1.4%, 95% CI 1.1–1.7) and rifampicin (1.4%, 95% CI 1.1–1.7). All isolates were considered as WT for vancomycin (Table 2). All isolates were considered as NWT as for at least four antimicrobials and to a mean of 8.83 antimicrobials ranging from 7.68 for broiler farms to 10.75 for dairy farms.

Microarray typing for resistance and virulence gene detection
Twenty-two isolates with different PFGE profiles at 80% similarity and different WT and NWT distributions were selected for microarray analysis. All tested isolates belonging to the NWT population for erythromycin carried at least one macrolide-lincosamide-streptogramin B resistance gene of the erm family. Six isolates carried **erm(A)**, nine carried **erm(B)** and four carried **erm(C)**. The frequency of NWT for clindamycin and quinupristin/dalfopristin was also high. This may be due to the presence of the erm genes since no isolates carried typical **S. aureus** lincosamide, pleuromutilin and streptogramin A resistance genes **vga(A)** and **vgb(A)**. However, other resistance genes not tested in our study might be involved. Only five isolates showed the **aacA-aphD** gene encoding aminoglycoside adenyl-/phosphotransferase. Six NWT isolates for kanamycin and three WT isolates were positive for the **aacD** aminoglycoside resistance gene. Among the 18 NWT isolates for tetracycline, 5 carried **tetK** and 16 **tet(M)**. All NWT isolates for chloramphenicol and one WT isolate carried the cat(pC221) or cat(pSBK203R) gene encoding chloramphenicol acetyltransferase. Ten isolates carried the lincosamide nucleotidyltransferase **lnu**(A) and all were resistant to the lincosamide clindamycin. The penicillin/ampicillin resistance operon **bla**(blaZ, **blaI** and **blaR**) was not detected. Five isolates carried the **qacC** efflux pump gene encoding resistance to quaternary ammonium disinfectants.

In addition to the resistance genes recovered, isolates were positive for several virulence genes. Genes encoding an immune evasion factor (**isaB**), staphylococcal superantigen-like proteins (**ssl10/set4**), a protease (**ssp**P) and site-specific deoxyribonucleases (**hdsS2** and **hdsSx**) were detected in >70% of the isolates. More than 25% of the isolates carried genes encoding a leucocidin (**lukS**), fibronectin-binding protein A (**fnbA**), an immune evasion factor (**isaD**) and a bone sialoprotein-binding protein (**bbp**). A biofilm-associated gene (**bap**) and genes encoding a putative transporter (**imrP**), bone sialoprotein-binding protein C (**sdrC**) and **S. aureus** surface protein **G** (**sasG**) were found in ~20% of the isolates. Other virulence genes such as the staphylococcal superantigen-like genes (**setB2** and **ssl03/set8**), the gene encoding the putative membrane protein (**hiIII**), the gene encoding enterotoxin (**sed**), the gene encoding the arginine catabolic mobile element (**ACME**) locus (**arcD-SCC**) and the gene encoding the capsule (**capK1**) were found in only one isolate.

Discussion
**S. sciuri** is an ancestral species within the genus **Staphylococcus**. Long considered as a commensal species, several clinical cases have now been reported in both human and veterinary medicine. However, little is known about its epidemiology and genetic diversity in healthy animals. This study aimed to estimate the prevalence of MRSS in different healthy farm animals and to determine its genetic diversity in order to assess its potential role as a reservoir of virulence and resistance genes for other staphylococci such as **S. aureus**.

We found 71 MRSS leading to a total prevalence of <9.5% for MRSS in healthy farm animals. This prevalence ranged from 3% for beef cows to around 10% for dairy cows and veal calves. The prevalence in broilers (12.5%) is quite low compared with that in

Table 1. MRSS prevalence and SCCmec distribution in different farm animals

<table>
<thead>
<tr>
<th>Farm animal (total number of animals tested)</th>
<th>Number of positive animals</th>
<th>Prevalence (%)</th>
<th>95% CI</th>
<th>SCCmec III (%)</th>
<th>SCCmec IIIA (%)</th>
<th>SCCmec NT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigs (200)</td>
<td>13</td>
<td>6.5</td>
<td>3.1–9.92</td>
<td>61.5</td>
<td>7.7</td>
<td>30.8</td>
</tr>
<tr>
<td>Dairy cows (100)</td>
<td>10</td>
<td>10.0</td>
<td>4.1–15.9</td>
<td>60.0</td>
<td>35.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Beef cows (100)</td>
<td>3</td>
<td>3.0</td>
<td>0.0–6.3</td>
<td>10.0</td>
<td>0.0</td>
<td>90.0</td>
</tr>
<tr>
<td>Veal calves (150)</td>
<td>20</td>
<td>13.3</td>
<td>7.9–18.8</td>
<td>33.3</td>
<td>0.0</td>
<td>66.7</td>
</tr>
<tr>
<td>Broilers (200)</td>
<td>25</td>
<td>12.5</td>
<td>7.9–17.1</td>
<td>92.0</td>
<td>8.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total (750)</td>
<td>71</td>
<td>9.5</td>
<td>7.4–11.6</td>
<td>63.4</td>
<td>14.1</td>
<td>22.5</td>
</tr>
</tbody>
</table>

NT, non-typeable.
a former study on MRSS in Belgium, which was estimated at 30% on a farm level. Other studies showed the presence of MRSS in different farm animals such as chickens, horses and dogs, though no accurate prevalence could be estimated. SCCmec analysis showed a higher prevalence of type III cassette, which accounted for 60% of the SCCmec recovered and was predominant in broilers as well as in dairy cows and pigs. This is in accordance with Zhang et al. who found SCCmec type III predominant in MRCoNS from animal origin. Non-typeable SCCmec were found in >20% of the isolates and were predominant in veal calves and beef cows. Though no ccr complex could be detected by the PCR described by Kondo et al. in the non-typeable SCCmec, only one of these isolates was negative for all ccr genes tested in the microarray. This was also observed in the study of MRSS in poultry in Belgium. This supports the hypothesis of the carriage of a ccrA element with an aberrant sequence that was proposed in this latter study. This high prevalence of non-typeable SCCmec also confirms the potential divergence of ccr and mec complexes in S. sciuri as suggested by Urushibara et al.
Table 2. MIC distribution of MRSS from farm animals

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Percentage of isolates with an MIC (mg/L) of:</th>
<th>Percentage resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;0.016</td>
<td>0.03</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>12.7</td>
<td>57.7</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>1.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>47.9</td>
<td>0.0</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>12.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kanamycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linezolid</td>
<td>36.6</td>
<td>7.0</td>
</tr>
<tr>
<td>Mupirocin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>1.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>88.7</td>
<td>7.0</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>1.4</td>
<td>7.0</td>
</tr>
<tr>
<td>Quinupristin/</td>
<td>38.0</td>
<td>0.0</td>
</tr>
<tr>
<td>dalfopristin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Tiamulin</td>
<td>21.1</td>
<td>47.9</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>94.4</td>
<td>5.6</td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Empty boxes indicate concentration values that were not tested. Values in grey boxes indicate MICs higher or lower than the concentrations tested. The vertical broken black lines indicate EUCAST ECOFFs for S. aureus. The vertical broken black lines indicate EUCAST ECOFFs for CoNS where different from those for S. aureus. MIC values were interpreted using EUCAST ECOFFs.

PFGE with Smal restriction was used here to assess diversification among isolates since this method has been shown to have high discriminative power in S. aureus. Some isolates were recovered from different species on the same farms. However, some of those isolates were separated among the different clusters observed. The different animal origins were also scattered among the different clusters. This indicates high diversity among the farms sampled and low host specificity. These results differ from those of the study on poultry in Belgium since, in the latter study, broiler and layer S. sciuri isolates were clearly separated in two different clusters.

Unexpectedly, not all isolates belonged to the NWT population for penicillin and cefoxitin in the broth microdilution tests, despite the use of selective media for MRSS isolation and the presence of the mecA gene in all isolates. However, since ECOFF are not available for those two antimicrobials in CoNS, this method might not be reliable in this case (http://www.eucast.org). In addition, the EUCAST ECOFFs for S. aureus and CoNS of clindamycin and trimethoprim do not seem to fit with the observed distribution. Indeed, a clear bimodal distribution was seen for clindamycin with an ‘NWT group’ (MIC >4 mg/L) and a ‘WT group’ (MIC <2 mg/L). However, we found resistance genes (erm(A), erm(B) or erm(C)) in all strains from both those groups. We are thus not able to propose better cut-off values for clindamycin. On the other hand, for trimethoprim, a clear bimodal distribution was seen with a ‘resistant group’ showing an MIC around 4 mg/L and a ‘susceptible group’ showing an MIC around 32 mg/L. Since the trimethoprim resistance genes dfrS1 (dfr(A)) were not detected, the resistance may be due to other dfr genes such as dfrD or dfrG, which were not included in the S. aureus genotyping kit and have previously been detected in members of the S. sciuri species group.

All isolates belonged to the NWT population for gentamicin using ECOFFs for CoNS. However, only isolates with an MIC ranging between 2 and 4 mg/L were positive for the gentamicin resistance gene aacA-aphD. Since the cut-off value to determine WT and NWT for this antimicrobial is ≏2 mg/L for S. aureus while it is ≏1 mg/L for CoNS, this indicates that the gentamicin ECOFFs for S. aureus may be more accurate than that of CoNS for S. sciuri. It is also to be noted that the microtitre plates used in this study do not allow the detection of MICs of <1 mg/L for gentamicin. It is thus not possible to differentiate between isolates with an MIC ≏1 mg/L while the ECOFF for CoNS is <0.5 mg/L. The frequency of NWT was very high for tiamulin and fusidic acid, though no resistance genes associated with those antimicrobials could be detected. This situation has previously been described by Frey et al., who suggested the possible appearance of new resistance genes or mutations in the elongation factor G. Few isolates were NWT for linezolid, which is in accordance with Gu et al., who found ~1.4% of the CoNS in hospital resistant to linezolid.

Several of our S. sciuri isolates harboured virulence genes such as sed and arcD, which are located on mobile genetic elements. These isolates may thus be regarded as a source of virulence genes for other staphylococci such as livestock-associated MRSA ST398, which currently carries few virulence genes. The presence of resistance and virulence genes similar...
to those found in S. aureus enhances the hypothesis that S. sciuri might be an important reservoir of these genes.

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Transparency declarations

None to declare.

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