High occurrence of methicillin-resistant Staphylococcus aureus ST398 in equine nasal samples

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Abstract

Methicillin-resistant Staphylococcus aureus (MRSA) infections do occur in equine patients. Little is known, however, about their origin and the general equine MRSA colonization status. In West European horses in particular, neither the colonization rate nor the present strains or their antimicrobial susceptibility patterns are known.

In the present study, a sample of 110 (Belgian, French, Dutch and Luxemburg) horses presented at a Belgian equine clinic was screened for nasal MRSA carriage. An indirect culturing protocol using a 0.001% colistin and nalidixic acid containing broth was compared to a direct agar method. Phenotypic identification following growth on a chromogenic MRSA screening agar (ChromID® MRSA) was combined with genotypic analysis (PCR, PFGE, SCCmec, spa, and MLST typing). Antimicrobial susceptibility was tested through disk diffusion.

Twelve (10.9%) horses carried MRSA, with the enrichment protocol resulting in a significantly higher isolation rate. None of the isolated strains were typeable through Smal PFGE. They all harboured SCCmec type IVa or V and belonged to spa type t011 or t1451 of the ST398 lineage. All isolates were tetracycline resistant and sulfonamide and enrofloxacin susceptible. Macrolide, lincosamide, trimethoprim and aminoglycoside susceptibility varied and in total five different antimicrobial resistance patterns were distinguished.

These results show that ST398 is certainly present in West European horses. Due to its known interspecies transmission and the structure of the equine industry, the presence of this clone in horses poses a substantial health hazard for both animals and humans.

Keywords: Staphylococcus aureus; MRSA; Horse; Nasal colonization, ST398

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1. Introduction

In recent years, methicillin-resistant *Staphylococcus aureus* (MRSA) has been isolated from several animal species (Lee, 2003; Goñi et al., 2004; Rich and Roberts, 2006; de Neeling et al., 2007) and the question of potential interspecies and zoonotic transmission has been raised (Weese et al., 2006a; Monecke et al., 2007). In horses it is known to cause varying types of infection in both colonized and non-colonized individuals (Shimizu et al., 1997; Weese et al., 2006b; Hermans et al., 2008).

The importance of MRSA colonization in the equine population is indeed twofold: colonized equids are at a higher risk of developing infections themselves and they can contaminate their environment, contact animals and humans (Weese et al., 2004, 2006a,b; Weese and Rousseau, 2005). Hence, equine MRSA colonization has important implications for both horse and man (Weese et al., 2006a). A high colonization and thus infection risk, incurred by large- animal personnel (colonization prevalence as high as 15.6%), has been reported (Weese et al., 2005; Hanselman et al., 2006). Vice versa, personnel and especially veterinarians may play an important role in the dissemination of the pathogen among animals (Anderson and Weese, 2006).

Up till now, most of the equine research has focused on American and Canadian horses and points to Canadian epidemic MRSA-5 (CMRSA-5) as the predominant equine strain (Seguin et al., 1999; Weese et al., 2005, 2006b). Little is known however about the European equine colonization status.

Due to the occurrence of a cluster of clinical MRSA infections at the institution of the first author (Hermans et al., 2008) questions were raised as to the source of patient contamination. Therefore, the aim of this study was to screen the arriving patient population to determine its colonization rate and the strain types involved. This is a first requirement for the epidemiologically and legally important differentiation between hospital-acquired versus hospital-expressed infections (Weese et al., 2006b). Determination of the isolates’ antimicrobial susceptibility patterns could also provide a first treatment directive in case of later infections. Furthermore, a first, although rough, estimate of the MRSA colonization status of West European equids would be provided.

2. Materials and methods

2.1. Sample collection

2.1.1. Study population

Nasal swabs were taken from 110 horses presented at the Department of Surgery and Anaesthesiology of Domestic Animals, Faculty of Veterinary Medicine, Ghent University, a tertiary referral centre receiving mostly Belgian, French, Dutch and Luxemburg equine patients. All horses arriving at the clinic between March and July 2007 were eligible for sampling. Four vets were charged with swabbing each patient within 1 h after arrival. Whenever immediate sampling was impossible or owner or patient compliance was not achieved, patients were excluded from further study. Both ambulatory and future hospitalised patients were screened, with no distinction being made for their reason of admission. For every ten patient samples a control sample was taken by inserting a swab into sterile saline (0.9% NaCl).

2.1.2. Sampling

For each horse, a cotton-tipped swab was fully (±12 cm) inserted into one nasal passage, retracted in contact with the nasal mucosa and embedded in Stuart’s medium (UNI-TER AMIES CLR, Piove di Sacco, Italy). After storage in the medium at 4 °C for a maximum of 24 h, the samples were preserved at −70 °C until further processing.

2.2. MRSA isolation and phenotypic identification

A total of 121 swabs was processed. After defrosting the samples in groups of 11 (including 1 control sample per 10 different patient samples), they were cultured. For the first 20 patient samples, both direct and enrichment culturing was used, for the remaining swabs, only the enrichment method was applied.

For direct culturing, swabs were inoculated onto a chromogenic MRSA screening agar (ChromID™ MRSA, bioMérieux, Lyon, France) and incubated aerobically for 48 h at 37 °C. Enrichment culturing entailed inoculation of a 5 ml medium, consisting of Brain Heart Infusion (BHI) broth containing 0.001% colistin and 0.001% nalidixic acid. After incubation for 24 h at 37 °C, a loopful was inoculated onto ChromID™ agar and incubated as described above.
Upon evaluation of the growth on ChromID™ agar after 24 and 48 h, according to the manufacturer’s recommendations, MRSA-suspicious colonies were purified on Columbia agar containing 5% sheep blood and colistin/nalidixic acid (Columbia CNA, Oxoid, Wesel, Germany) for 24 h at 37 °C. Identification of S. aureus was based on colony morphology, haemolytic properties, DNase and catalase activity and specific growth on a modified Baird–Parker agar (Devriese, 1981).

2.3. Genotypic identification of MRSA presence

For the detection of mecA (methicillin resistance) and femA (S. aureus specific) genes, a multiplex PCR was performed using primers described by Mehrotra et al. (2000). For extraction of the DNA, one colony of each isolate was suspended in 20 µl of a lysis buffer (0.25% SDS, 0.05 N NaOH) and heated at 95 °C for 5 min. The samples were centrifuged briefly at 16,000 × g at room temperature and diluted by adding 180 µl of distilled water. Consequently, a centrifugation for 5 min at 16,000 × g was performed to remove cell debris. Supernatants were then frozen at −20 °C until further use.

Subsequent DNA amplification was performed using a thermal cycler (Biometra, Gottingen, Germany). Each amplification reaction was performed in a 30 µl reaction volume containing 2 mM MgCl2, 1.5 U taq DNA polymerase, 0.2 mM of dNTP’s, 0.66 µM of each of four primers and 6 µl of the DNA sample. Consequently, agarose gel electrophoresis using 5 µl of amplicon mixed with 3 µl of sample buffer (50% glycerol, 1 mM cresol Red) was followed by visualization photography under UV light. The gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas, St. Leon-Rot, Germany) was used as a DNA size marker.

Two mecA positive, femA negative strains, which grew on the ChromID™ agar, were identified further through rRNA intergenic spacer PCR, according to Baele et al. (2000).

2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method. Tetracycline, enrofloxacin, erythromycin, tylosin, clindamycin, lincomycin, sulfonamide, trimethoprim, gentamicin and neomycin resistance was tested through incubation (37 °C, 24 h) with Neo-Sensitabs™ (Rosco Diagnostica A/S, Taastrup, Denmark) on Iso-Sensitest agars (Oxoid, Wesel, Germany). Oxacillin resistance was similarly tested with incubation at 30 °C for 48 h. Inoculum standardization, medium and incubation conditions as well as interpretation of inhibition zones were performed according to the tablet manufacturer’s guidelines (Guidelines for the use of Neosensitabs™, 18th ed., 2005/2006, http://www.rosco.dk).

2.5. Genotyping

Further characterization of isolates involved SmaI pulsed-field gel electrophoresis (PFGE) according to the protocol of Deplano et al. (2000), followed by spa typing (single locus DNA sequencing of the X region of the protein A gene) (Harmsen et al., 2003) and SCCmec typing as described by Oliveira and de Lencastre (2002) and Zhang et al. (2005). Multilocus sequence typing (MLST) (Enright et al., 2000) was performed on three representative MRSA strains (Table 1).

2.6. Statistics

All statistical analyses were performed using the statistical software program SPSS 15.0 (SPSS Inc., Chicago, USA). The direct and indirect culturing methods were compared using a McNemar’s exact test with calculation of the kappa value. The presence of association between a horse revisiting the clinic and being MRSA positive was investigated using a χ²-test and odds ratio. In addition, a Fisher’s exact test for R*C tables was used to test whether the country of origin of a horse was correlated with its colonization status. A significance level (α) of 0.05 was set for all analyses.

3. Results

Of the 850 horses arriving at the clinic during the study period 110 (12.9%) were swabbed. The obtained samples originated from 80 (72.7%) Belgian, 24 (21.8%) French (Departments Pas-de-Calais, Nord,
Somme), 3 (2.7%) Dutch (province Zeeland) and 3 (2.7%) Luxemburg horses. Eighty-nine (80.9%) of them were first time visits, 21 (19.1%) had already been at the clinic. One horse that came from a different department in the same clinic was considered to be a readmission. For the others the time frame between previous discharge and readmission varied between 1 week and 2 years. Only on three occasions did two horses come from the same housing facility and only one of those was positive.

Twelve out of the 110 patients arriving at the clinic were identified as nasal MRSA carriers (proportion 0.109 with 95% confidence interval [0.051, 0.167]). Since one of them tested positive on both the direct and indirect culturing method, a total of thirteen isolates was obtained (Table 1). Only one of the colonized horses had a MRSA containing wound infection, which was already present upon arrival. The others were subclinical carriers. All positive horses came from different farms. Eight of the positive samples originated from horses arriving in March 2007, 2 were gathered in May and 2 in June 2007. In each of these periods both French and Belgian horses tested positive. Neither prior visits at the same clinic ($\chi^2$-test, $P = 1.00$), nor the horses’ country of origin (RxC table using Fisher’s exact tests, $P = 0.73$) were associated with the patient’s colonization status.

The indirect culturing protocol resulted in a significantly higher isolation rate than the direct method. (McNemar’s exact test, $P = 0.03$, $\kappa = 0.18$). All but one of the isolates appeared on the ChromIDTM agar after 24 h incubation. Forty-eight hour incubation led to one additional isolate and 24 false positives. The latter included two mecA positive, femA negative isolates, which had a doubtful morphology on the modified Baird–Parker medium and were identified through tRNA intergenic spacer PCR as being coagulase negative $S. sciuri$.

All MRSA isolates contained the SCCmec elements IVa or V, were non-typeable through PFGE using SmaI digestion and belonged to spa types t011 and t1451 (Table 1). This characterizes them as ST398 (de Neeling et al., 2007; Witte et al., 2007), which was confirmed by MLST typing of three representative strains (Table 1). For the only patient testing positive through both the direct and enrichment protocol, two spa types, differing by one repeat deletion and demonstrating identical resistance patterns, SCCmec and MLST types, were isolated (Table 1). The isolates’

Table 1  
Resistance patterns of equine nasal ST398 isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origina</th>
<th>PFGE-SCCmec-spa</th>
<th>spa profile</th>
<th>MLST</th>
<th>Antimicrobial agentb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>oxa</td>
</tr>
<tr>
<td>AV4</td>
<td>Belgian</td>
<td>Nt\textsuperscript{c}-IVa-t011</td>
<td>8-16-2-25-34-24-25</td>
<td>ST398</td>
<td>R</td>
</tr>
<tr>
<td>AV4\textsuperscript{d}</td>
<td>Belgian</td>
<td>Nt\textsuperscript{-}IVA-t1451</td>
<td>8-16-2-25-34-25</td>
<td>ST398</td>
<td>R</td>
</tr>
<tr>
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<td>Belgian</td>
<td>Nt-IVA-t011</td>
<td>8-16-2-25-34-25</td>
<td>ST398</td>
<td>R</td>
</tr>
<tr>
<td>AV6</td>
<td>Belgian</td>
<td>Nt-V-t011</td>
<td>8-16-2-25-34-25</td>
<td>ST398</td>
<td>R</td>
</tr>
<tr>
<td>AV7</td>
<td>Belgian</td>
<td>Nt-V-t011</td>
<td>8-16-2-25-34-25</td>
<td>ST398</td>
<td>R</td>
</tr>
<tr>
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<td>Belgian</td>
<td>Nt-IVA-t011</td>
<td>8-16-2-25-34-25</td>
<td>ST398</td>
<td>R</td>
</tr>
<tr>
<td>AV18</td>
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<td>Nt-IVA-t011</td>
<td>8-16-2-25-34-25</td>
<td>ST398</td>
<td>R</td>
</tr>
<tr>
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<td>French</td>
<td>Nt-IVA-t011</td>
<td>8-16-2-25-34-25</td>
<td>ST398</td>
<td>R</td>
</tr>
<tr>
<td>AV24</td>
<td>Belgian</td>
<td>Nt-IVA-t011</td>
<td>8-16-2-25-34-25</td>
<td>ST398</td>
<td>R</td>
</tr>
<tr>
<td>AV63</td>
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<td>Nt-IVA-t011</td>
<td>8-16-2-25-34-25</td>
<td>ST398</td>
<td>R</td>
</tr>
<tr>
<td>AV78</td>
<td>French</td>
<td>Nt-IVA-t011</td>
<td>8-16-2-25-34-25</td>
<td>ST398</td>
<td>R</td>
</tr>
<tr>
<td>AV84</td>
<td>French</td>
<td>Nt-IVA-t011</td>
<td>8-16-2-25-34-25</td>
<td>ST398</td>
<td>R</td>
</tr>
<tr>
<td>AV91</td>
<td>Belgian</td>
<td>Nt-IVA-t011</td>
<td>8-16-2-25-34-25</td>
<td>ST398</td>
<td>R</td>
</tr>
</tbody>
</table>

| oxo, oxacillin; tet, tetracycline; tri, trimethoprim; gen, gentamicin; neo, neomycin; ery, erythromycin; tyl, tylosin; lin, lincomycin; cli, clindamycin; sul, sulfonamides; enr, enrofloxacin. |
|a Horse’s country of origin. |
|b R, resistant; S, sensitive. |
|c Nt, non-typeable through SmaI digestion. |
d Same swab as AV4 but cultured using the direct protocol. |
antimicrobial susceptibility tests (Table 1) revealed five different patterns.

4. Discussion

The aim of this study was to determine if and in what percentage MRSA could be isolated from the equine population arriving at a West European equine referral facility. In addition, each isolate was genetically typed and its antimicrobial susceptibility tested.

Compared to the 2.7% equine (CMRSA-5) carriers arriving at a Canadian clinic (Weese et al., 2006b), the proportion found in the present study (10.9%) is both astonishing and disturbing. Several factors, such as a high regional MRSA prevalence at the time of the investigation, a sampling bias, the colonizing strain type and or the isolation method used could have caused this.

Surprisingly, all strains isolated from the examined horses belonged to a clonal population which is currently being detected in different animal species and their caretakers (Witte et al., 2007; de Neeling et al., 2007; Monecke et al., 2007). ST398 colonization in horses, however, had not been established prior to this study. Consequently, equine infections with the latter clone were formerly considered to be of human origin (Witte et al., 2007). Yet, after three clinical ST398 infections in Austrian and German equids (Witte et al., 2007) and a clinical ST398 cluster of infections occurring at the first author’s clinic (Hermans et al., 2008), the high colonization percentage (10.9%) found in this study seems to advocate the current endemic status of ST398 in West European horses, with a likely transmission outside the clinical environment. A sampling bias due to admission to a tertiary referral clinic cannot be ruled out, though.

Interspecies transmission of S. aureus ST398 with exchange between animals and their caretakers has been demonstrated (Voss et al., 2005; de Neeling et al., 2007). Since the detection of both SCCmec types IVa and V in this study contradicts the potential independent evolution of equine ST398 proposed by Witte et al. (2007), there is no reason to assume that horses would not take part in the interspecies transmission. The emergence of the clone as an equine nasal colonist might even pose a greater threat than its presence in intensive animal production. Indeed, due to the current international spread of the equine industry and broad base of passionate horse-lovers, equids may form an important and continuous reservoir for the spread of ST398. Occupational or recreational horse exposure has already been incriminated as an important risk factor for human MRSA colonization or infection (Weese et al., 2006a).

As stated above, the high isolation rate reported in this research paper can (partly) be due to the isolation protocol used. Given the expected polybacterial nature of equine nasal mucosae and the detection limit of direct bacterial culturing, the use of an enrichment broth was desirable in this study. Due to a lack of studies comparing animal MRSA isolation protocols the author’s choice was based on human literature. Hence, a 0.001% colistin and nalidixic acid BHI solution was preferred to the equine more commonly used high salt concentration enrichment broths (4% and 7.5%) (Busscher et al., 2006; Weese et al., 2006b) since the latter are known to inhibit growth of several human MRSA strains (Bruins et al., 2007). After this enhancement of Gram positive growth, commercial cefoxitin containing chromID™ MRSA (formerly called MRSA ID) agars were used to enhance selectivity because, at least for human isolates, the equine more frequently used mannitol salt agars containing oxacillin show a lower sensitivity (Perry et al., 2004; Weese et al., 2004, 2006b). The same was suspected for the CHROMagar, used in horses by Cuny et al. (2006), especially in the presence of competing nasal flora (Perry et al., 2004). The applicability of the protocol was certainly demonstrated in this study. Further research as to the value of different equine screening protocols is warranted though, bearing in mind that the harmonization of screening protocols would enhance the comparability between studies, although, it might decrease the diversity of isolated strain types.

The antimicrobial resistance pattern detected in the equine isolates (Table 1) does not coincide with the current antimicrobial usage in equids (Prescott et al., 2006). In addition, its variability shows that antimicrobial susceptibility testing can be a valid asset to epidemiological strain tracing. The quick availability of such data can be advantageous during clinical outbreaks, from both a clinical and epidemiological point of view. Moreover, the observed diversity
confirms that also in equine infections not responding to first line antimicrobial therapy, bacteriological examination with antimicrobial susceptibility testing should be conducted, in order to speed the onset of appropriate therapy and limit resistance formation and selection of resistant species.

5. Conclusion

In conclusion, we can state that the proposed protocol was successful in screening an equine population for MRSA colonization. It demonstrated that the MRSA clone ST398 has become a nasal opportunistic pathogen in West European horses with a plausible zoonotic potential. Finally, antimicrobial susceptibility testing can be an important tool from both a therapeutic and epidemiologic point of view.

References


