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1 **High occurrence of methicillin-resistant *Staphylococcus aureus* ST398 in**
2 **equine nasal samples.**

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20 **ABSTRACT**

21

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

26 In the present study, a sample of 110 (Belgian, French, Dutch and Luxemburg) horses presented at a
27 Belgian equine clinic was screened for nasal MRSA carriage. An indirect culturing protocol using a
28 0.001% colistin and nalidixic acid containing broth was compared to a direct agar method. Phenotypic
29 identification following growth on a chromogenic MRSA screening agar (ChromID™ MRSA) was
30 combined with genotypic analysis (PCR, PFGE, *SCCmec*, *spa*, and MLST typing). Antimicrobial
31 susceptibility was tested through disk diffusion.

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

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

41

42 **KEYWORDS**43 *Staphylococcus aureus*, MRSA, horse, nasal colonization, ST398

44 **1. Introduction**

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

50 The importance of MRSA colonization in the equine population is indeed twofold: colonized equids
51 are at a higher risk of developing infections themselves and they can contaminate their environment,
52 contact animals and humans (Weese et al., 2004; Weese and Rousseau 2005; Weese et al. 2006a;
53 2006b). Hence, equine MRSA colonization has important implications for both horse and man (Weese
54 et al., 2006a). A high colonization and thus infection risk, incurred by large-animal personnel
55 (colonization prevalence as high as 15.6%), has been reported (Weese et al., 2005; Hanselman et al.,
56 2006). Vice versa, personnel and especially veterinarians may play an important role in the
57 dissemination of the pathogen amongst animals (Anderson and Weese, 2006).

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

61 Due to the occurrence of a cluster of clinical MRSA infections at the institution of the first author
62 (Hermans et al., in press) questions were raised as to the source of patient contamination. Therefore,
63 the aim of this study was to screen the arriving patient population to determine its colonization rate
64 and the strain types involved. This is a first requirement for the epidemiologically and legally
65 important differentiation between hospital-acquired versus hospital-exposed infections (Weese et al.,
66 2006b). Determination of the isolates' antimicrobial susceptibility patterns could also provide a first
67 treatment directive in case of later infections. Furthermore, a first, although rough, estimate of the
68 MRSA colonization status of West European equids would be provided.

69

70 **2. Materials and Methods**

71

72 **2.1. Sample Collection**

73

74 *2.1.1. Study population*

75

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

85

86 *2.1.2. Sampling*

87

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

92

93 **2.2 MRSA isolation and phenotypic identification**

94

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

99 For direct culturing, swabs were inoculated onto a chromogenic MRSA screening agar (ChromID™
100 MRSA, bioMérieux, Lyon, France) and incubated aerobically for 48 hrs at 37 °C. Enrichment
101 culturing entailed inoculation of a 5 ml medium, consisting of Brain Heart Infusion (BHI) broth
102 containing 0.001% colistin and 0.001% nalidixic acid. After incubation for 24 hrs at 37 °C, a loopful
103 was inoculated onto ChromID™ agar and incubated as described above.

104 Upon evaluation of the growth on ChromID™ agar after 24 and 48 hrs, according to the
105 manufacturer's recommendations, MRSA-suspicious colonies were purified on Columbia agar
106 containing 5% sheep blood and colistin/nalidixic acid (Columbia CNA, Oxoid, Wesel, Germany) for
107 24 hrs at 37°C. Identification of *S. aureus* was based on colony morphology, haemolytic properties,
108 DNase and catalase activity and specific growth on a modified Baird-Parker agar (Devriese, 1981).

109

110 **2.3. Genotypic identification of MRSA presence**

111

112 For the detection of *mecA* (methicillin resistance) and *femA* (*S. aureus* specific) genes, a multiplex
113 PCR was performed using primers described by Mehrotra et al. (2000). For extraction of the DNA,
114 one colony of each isolate was suspended in 20 µl of a lysis buffer (0.25% SDS, 0.05 N NaOH) and
115 heated at 95°C for 5 minutes. The samples were centrifuged briefly at 16000 g at room temperature
116 and diluted by adding 180 µl of distilled water. Consequently, a centrifugation for 5 minutes at 16000
117 g was performed to remove cell debris. Supernatants were then frozen at -20°C until further use.

118 Subsequent DNA amplification was performed using a thermal cycler (Biometra, Gottingen,
119 Germany). Each amplification reaction was performed in a 30 µl reaction volume containing 2 mM
120 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
121 the DNA sample. Consequently, agarose gel electrophoresis using 5 µl of amplicon mixed with 3 µl of
122 sample buffer (50% glycerol, 1mM cresol Red) was followed by visualisation photography under UV
123 light. The gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas, St. Leon-Rot, Germany) was used
124 as a DNA size marker.

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

127

128 **2.4. Antimicrobial susceptibility testing**

129

130 Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method.
131 Tetracycline, enrofloxacin, erythromycin, tylosin, clindamycin, lincomycin, sulfonamide,
132 trimethoprim, gentamicin and neomycin resistance was tested through incubation (37 °C, 24 hrs) with
133 Neo-Sensitabs™ (Rosco Diagnostica A/S, Taastrup, Denmark) on Iso-Sensitest agars (Oxoid, Wesel,
134 Germany). Oxacillin resistance was similarly tested with incubation at 30°C for 48 hrs. Inoculum
135 standardization, medium and incubation conditions as well as interpretation of inhibition zones were
136 performed according to the tablet manufacturer's guidelines (Guidelines for the use of Neosensitabs™,
137 18th ed., 2005/2006, <http://www.rosco.dk>).

138

139 **2.5. Genotyping**

140

141 Further characterisation of isolates involved *Sma*I pulsed-field gel electrophoresis (PFGE) according
142 to the protocol of Deplano et al. (2000), followed by *spa* typing (single locus DNA sequencing of the
143 X region of the protein A gene) (Harmsen et al., 2003) and SCC*mec* typing as described by Oliveira
144 and de Lencastre (2002) and Zhang et al. (2005). Multilocus sequence typing (MLST) (Enright et al.,
145 2000) was performed on three representative MRSA strains (Table 1).

146

147 **2.6. Statistics**

148

149 All statistical analyses were performed using the statistical software program SPSS 15.0 (SPSS INC,
150 Chicago, USA). The direct and indirect culturing methods were compared using a McNemar's exact
151 test with calculation of the kappa value. The presence of association between a horse revisiting the

152 clinic and being MRSA positive was investigated using a χ^2 -test and odds ratio. In addition, a Fisher's
153 exact test for R*C tables was used to test whether the country of origin of a horse was correlated with
154 its colonization status. A significance level (α) of 0.05 was set for all analyses.

155

156 **3. Results**

157

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

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

175 The indirect culturing protocol resulted in a significantly higher isolation rate than the direct method.
176 (McNemar's exact test, $P = 0.03$, $\kappa = 0.18$). All but one of the isolates appeared on the ChromID™
177 agar after 24h incubation. Forty eight hour incubation led to one additional isolate and 24 false
178 positives. The latter included two *mecA* positive, *femA* negative isolates, which had a doubtful

179 morphology on the modified Baird-Parker medium and were identified through tRNA intergenic
180 spacer PCR as being coagulase negative *S. sciuri*.

181 All MRSA isolates contained the SCC*mec* elements IVa or V, were non-typeable through PFGE using
182 *Sma*I digestion and belonged to *spa* types t011 and t1451 (Table 1). This characterizes them as ST398
183 (de Neeling, 2007; Witte et al., 2007), which was confirmed by MLST typing of three representative
184 strains (Table 1). For the only patient testing positive through both the direct and enrichment protocol,
185 two *spa* types, differing by one repeat deletion and demonstrating identical resistance patterns,
186 SCC*mec* and MLST types, were isolated (Table 1). The isolates' antimicrobial susceptibility tests
187 (Table 1) revealed five different patterns.

188

189 **4. Discussion**

190

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

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

198 Surprisingly, all strains isolated from the examined horses belonged to a clonal population which is
199 currently being detected in different animal species and their caretakers (Witte et al., 2007; de Neeling
200 et al., 2007; Monecke et al., 2007). ST398 colonization in horses, however, had not been established
201 prior to this study. Consequently, equine infections with the latter clone were formerly considered to
202 be of human origin (Witte et al., 2007). Yet, after three clinical ST398 infections in Austrian and
203 German equids (Witte et al., 2007) and a clinical ST398 cluster of infections occurring at the first
204 author's clinic (Hermans et al., in press), the high colonization percentage (10.9%) found in this study
205 seems to advocate the current endemic status of ST398 in West European horses, with a likely

206 transmission outside the clinical environment. A sampling bias due to admission to a tertiary referral
207 clinic cannot be ruled out, though.

208 Interspecies transmission of *S. aureus* ST398 with exchange between animals and their caretakers has
209 been demonstrated (Voss et al., 2005; de Neeling, 2007). Since the detection of both SCCmec types
210 IVa and V in this study contradicts the potential independent evolution of equine ST398 proposed by
211 Witte et al. (2007), there is no reason to assume that horses wouldn't take part in the interspecies
212 transmission. The emergence of the clone as an equine nasal colonist might even pose a greater threat
213 than its presence in intensive animal production. Indeed, due to the current international spread of the
214 equine industry and broad base of passionate horse-lovers, equids may form an important and
215 continuous reservoir for the spread of ST398. Occupational or recreational horse exposure has already
216 been incriminated as an important risk factor for human MRSA colonization or infection (Weese et al.,
217 2006a).

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

234 The antimicrobial resistance pattern detected in the equine isolates (Table1) does not coincide with the
235 current antimicrobial usage in equids (Prescott et al., 2006). In addition, its variability shows that
236 antimicrobial susceptibility testing can be a valid asset to epidemiological strain tracing. The quick
237 availability of such data can be advantageous during clinical outbreaks, from both a clinical and
238 epidemiological point of view. Moreover, the observed diversity confirms that also in equine
239 infections not responding to first line antimicrobial therapy, bacteriological examination with
240 antimicrobial susceptibility testing should be conducted, in order to speed the onset of appropriate
241 therapy and limit resistance formation and selection of resistant species.

242

243 **5. Conclusion**

244

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

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343 **TABLE**

344 Table 1.

345 Resistance patterns of equine nasal ST398 isolates

Isolate	Origin ^b	PFGE-SCC <i>mec-spa</i>	<i>spa</i> profile	MLST	Antimicrobial agent ^d										
					oxa	tet	tri	gen	neo	ery	tyl	lin	cli	sul	enr
AV4	Belgian	Nt ^c -IVa-t011	8-16-2-25-34-24-25	ST398	R	R	R	R	R	S	S	S	S	S	S
AV4 ^a	Belgian	Nt-IVa-t1451	8-16-2-25-34-25	ST398	R	R	R	R	R	S	S	S	S	S	S
AV5	Belgian	Nt-IVa-t011	8-16-2-25-34-24-25		R	R	R	R	R	S	S	S	S	S	S
AV6	Belgian	Nt-V-t011	8-16-2-25-34-24-25	ST398	R	R	S	S	S	S	S	S	S	S	S
AV7	Belgian	Nt-V-t011	8-16-2-25-34-24-25		R	R	S	S	S	S	S	S	S	S	S
AV10	Belgian	Nt-IVa-t011	8-16-2-25-34-24-25		R	R	R	R	S	S	S	S	S	S	S
AV18	Belgian	Nt-IVa-t011	8-16-2-25-34-24-25		R	R	R	R	S	R	R	R	R	S	S
AV19	French	Nt-IVa-t011	8-16-2-25-34-24-25		R	R	R	R	S	S	S	S	S	S	S
AV24	Belgian	Nt-IVa-t011	8-16-2-25-34-24-25		R	R	R	R	S	S	S	S	S	S	S
AV63	French	Nt-IVa-t011	8-16-2-25-34-24-25		R	R	R	R	R	R	R	R	R	S	S
AV78	French	Nt-IVa-t011	8-16-2-25-34-24-25		R	R	R	R	R	S	S	S	S	S	S
AV84	French	Nt-IVa-t011	8-16-2-25-34-24-25		R	R	R	R	S	R	R	R	R	S	S
AV91	Belgian	Nt-IVa-t011	8-16-2-25-34-24-25		R	R	R	R	S	R	R	R	R	S	S

346 ^a = same swab as AV4 but cultured using the direct protocol347 ^b = horse's country of origin348 ^c Nt = non-typeable through *Sma*I digestion349 ^d **R**= resistant, **S** = sensitive350 **oxa** = oxacillin, **tet** = tetracycline, **tri** = trimethoprim, **gen** = gentamicin, **neo** = neomycin, **ery** = erythromycin, **tyl** = tylosin, **lin** = lincomycin, **cli** =351 clindamycin, **sul** = sulfonamides, **enr** = enrofloxacin