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1 **Clonal spread of methicillin-resistant coagulase-negative staphylococci**
2 **among horses, personnel and environmental sites at equine facilities**

3

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7

8 **Running head:** Methicillin-resistant coagulase-negative staphylococci at equine facilities

9

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13

14 **Abstract**

15 A cross-sectional study was carried out to investigate species distribution and clonality of
16 methicillin-resistant coagulase-negative staphylococci (MRCoNS) isolated from the nasal
17 cavity of 39 horses and 32 veterinary or caretaker staff, and from 76 environmental surfaces at
18 three equine facilities in Denmark. MRCoNS obtained by selective isolation were
19 characterized by 16S rDNA sequencing, PCR detection of *mecA*, and pulsed-field gel
20 electrophoresis (PFGE). MRCoNS were found in 32 (82%) horses, 20 (63%) humans and 50
21 (66%) environmental samples. The most common species was *S. vitulinus* (n=56), followed
22 by *S. sciuri* (n=25), *S. haemolyticus* (n=14). Undistinguishable PFGE patterns were observed
23 in MRCoNS of equine, human and environmental origin. A *S. vitulinus* clone and a *S.*
24 *haemolyticus* clone were isolated from multiple horses, staff members and environmental sites
25 within a farm and a veterinary hospital, respectively. The results indicated that methicillin-
26 resistant strains of these two species can be shared by and possibly exchanged between horses
27 and personnel, either directly or through contaminated environments.

28
29 **Keywords:** antibiotic resistance, commensal bacteria, veterinary hospitals, contamination

30 **Introduction**

31 Coagulase-negative staphylococci (CoNS) comprise a diverse group of commensals found on
32 the skin and mucosae in both animals and humans but some species are recognised as
33 important opportunistic pathogens in humans (Martins and Cunha, 2007). *S. epidermidis* and
34 *S. haemolyticus* are the two most important human pathogenic species, mainly associated with
35 post surgical infections, indwelling and implanted foreign bodies (Kloos and Bannerman,
36 1994). The role of CoNS as veterinary pathogens is less known but some species have been
37 implicated in mastitis, wound infections and skin abscesses in various animal species (van
38 Duijkeren et al., 2004; Taponen et al., 2006). In a Danish study (Winther et al., 2008), CoNS
39 were cultured from 14% (16/114) clinical specimens from horses, including wound,
40 respiratory, ocular and uterine infections.

41
42 Methicillin-resistant staphylococci, in particular methicillin-resistant *S. aureus* (MRSA), are
43 resistant bacteria of great concern to public health. Methicillin resistance is more common in
44 CoNS than in *S. aureus* and approximately 50-90% of human clinical isolates are reported as
45 being resistant (von Eiff et al., 2000; Martins and Cunha, 2007). Methicillin-resistant CoNS
46 (MRCoNS) are common inhabitants on the nasal mucosa of healthy animals, especially horses
47 (Yasuda et al., 2000; Vengust et al., 2006; Bacgicil et al., 2007). In this cross-sectional study,
48 the objectives were i) to determine whether MRCoNS clones can be shared by horses and
49 veterinary or caretaker staff; ii) to identify the species involved; and iii) to assess MRCoNS
50 contamination in the environment of equine facilities.

51

52 **Materials and Methods**

53 *Sampling*

54 The sampling sites were two equine referral hospitals on two Danish islands (Hospital A on
55 Zealand and Hospital B on Funen) and one equine farm owned by the Danish Army. Human
56 participation in the study was on a strict voluntary basis and personal information was kept
57 anonymous. The 32 humans tested were healthy and not on antibiotic therapy at the time of
58 sampling. Animal samples were randomly collected from all horses at the farm (n=20) and
59 from accessible patients admitted at Hospitals A (n=9) and B (n=10). Horses at the farm were
60 clinically healthy and not on antibiotic therapy at the time of sampling. Most patients at the
61 two hospitals were subjected to antibiotic therapy. All samples were collected on a single day
62 at each facility. Cotton swabs were inserted into each nostril, rolled on the mucosal
63 membranes for 5 sec, and stored in Stuarts' medium (Statens Serum Institut, Copenhagen,
64 Denmark) prior to laboratory analysis. Environmental swabs were collected from 50 objects
65 handled by the staff, such as sinks and taps, stable doors, equine handling equipment, door
66 and cupboard handles, surgical equipment, computer keyboards and telephones, treatment
67 tables, kettles and radios.

68

69 *MRCoNS isolation and identification*

70 Methicillin-resistant staphylococci were detected after overnight enrichment in MRSA
71 selective broth (Statens Serum Institute, Copenhagen, Denmark) at 37°C, followed by plating
72 on Oxacillin Resistance Screening Agar Base (ORSAB, Oxoid, Hampshire, United Kingdom).
73 After 24-48 hr incubation at 35°C, a single presumptive methicillin-resistant staphylococcal
74 colony (blue or white on ORSAB) was isolated from each sample and confirmed as
75 methicillin-resistant by PCR detection of *mecA* (Zhang et al., 2004). Diversity of *mecA*-
76 positive isolates was assessed by Random Amplification of Polymorphic DNA (RAPD)
77 analysis (Bagegil et al., 2007) and isolates exhibiting distinct RAPD profiles were identified
78 by partial 16S rDNA sequencing using primers 1AF: 5'- AGAGTTTGATYMTGGCT-3' and

79 519R: 5'-GTRTTACCGCGGCTGCTG-3'.. Nucleotide sequences were compared with those
80 on Genbank and sequences with the highest similarity score to a type strain was deemed the
81 species identity.

82

83 *PFGE analysis*

84 PFGE was performed according to Murchan et al. (2003) with minor modifications (Bagcigil
85 et al., 2007). A low-range PFGE Marker (New England Biolabs, Ipswich, England) was used
86 as a molecular weight marker and for normalisation between gels. Gels were analysed using
87 GelCompar II software (Applied Maths, Kortrijk, Belgium), and cluster analysis was
88 performed by UPGMA based on the Dice similarity coefficient, with optimization and
89 position tolerance set at 0.5% and 1.5%, respectively. A similarity cut-off value of 90% was
90 used to define PFGE types. Within each PFGE type, isolates exhibiting minor band
91 differences were assigned to distinct subtypes.

92

93 **Results**

94 MRCoNS were isolated from 32 (82%) horses, 20 (63%) humans and 50 (66%)
95 environmental samples (Table 1). We identified 12 RAPD types and 16S rDNA sequencing of
96 isolates representative of each type revealed the occurrence of six species: *S. vitulinus* (n=56),
97 *S. sciuri* (n=25), *S. haemolyticus* (n=14), *S. equorum* (n=4), *S. epidermidis* (n=2) and *S.*
98 *saprophyticus* (n=1). *S. vitulinus* was the most common MRCoNS species in horses (46%),
99 humans (28%) and the environment (38%). The species distribution differed greatly between
100 the three equine facilities. *S. vitulinus* accounted for 80% and 70% of MRCoNS isolates from
101 the equine farm and Hospital B, respectively, but was less frequent among isolates from
102 Hospital A (22%). *S. haemolyticus* and *S. sciuri* was isolated more frequently at Hospital A
103 (30% and 49%, respectively) than at the other two sampling sites (< 10%). *S. epidermidis* and

104 *S. saprophyticus* were only isolated from humans at the farm. *S. equorum* was only isolated at
105 Hospital B, from a horse, a veterinarian and samples from the stall and radio. MRCoNS were
106 isolated from all environmental sites and the most contaminated sites were wash and treatment
107 areas (Table 1).

108
109 High variability of PFGE patterns was observed within the three major species. The degree of
110 inter-species diversity in band patterns was notably greater for *S. vitulinus* (up to 60% and
111 58%, respectively) than for *S. haemolyticus* (up to 25%). Twenty-four distinct PFGE types
112 were identified among the 56 *S. vitulinus* isolates (See Figure S1, supplementary file). A
113 dominant *S. vitulinus* clone (Type A) was isolated from four horses, one human and various
114 environmental sites within the farm, including wash areas, door handles, horse restraints, and
115 stall bars (Figure 1a). Other less frequent indistinguishable PFGE patterns were shared
116 between equine or human and environmental *S. vitulinus* isolates from the same equine
117 facility. One indistinguishable *S. vitulinus* clone (Type E) was isolated from a veterinarian at
118 Hospital B and a horse and stall bar at the Farm. At Hospital A, clonal spread of *S.*
119 *haemolyticus* and *S. sciuri* was observed. Detection of the *S. sciuri* clone was limited to
120 equine and environmental samples, whereas the *S. haemolyticus* clone occurred in all sample
121 types (Figures 1b-c). The four *S. equorum* isolates from Hospital B and the two *S. epidermidis*
122 isolates from the farm displayed genetically unrelated PFGE patterns (data not shown).

123

124 **Discussion**

125 MRCoNS were widely disseminated among horses, humans and environmental sites at the
126 three equine facilities. The PFGE results demonstrated that specific methicillin-resistant *S.*
127 *vitulinus* and *S. hamolyticus* clones were shared by various horses and humans with the same
128 farm or hospital environment, indicating possible interspecies transmission, either directly or

129 through the contaminated environment. Although methicillin-resistance in staphylococci is a
130 public health concern, not all staphylococcal species are equally important as human or
131 veterinary pathogens. *S. haemolyticus* is one of the most important pathogenic species among
132 CoNS isolated from human infections (Kloos and Bannerman, 1994). This species is also
133 sporadically isolated from equine infections (van Duijkeren et al., 2004). On the contrary, *S.*
134 *vitulinus* has not been associated with human nor animal infections.

135

136 Although clone sharing suggests that MRCoNS transmission between horses and humans may
137 have occurred at two of the three equine facilities, the present study cannot be used to infer
138 the direction of transmission. However, some speculations can be made based on the natural
139 habitats of the species involved. Methicillin-resistant *S. vitulinus* is known to be a frequent
140 commensal in the nasal cavity of horses (Bagcigil et al., 2007) and is not associated with
141 carriage in healthy humans (Nagase et al., 2002; Busscher et al., 2006). Therefore, the *S.*
142 *vitulinus* clone isolated from humans is likely to have originated from the horses or from the
143 contaminated environment. In contrast, the *S. haemolyticus* clone at Hospital A could have
144 originated from either horses or humans since this species is part of the normal nasal flora of
145 both hosts.

146

147 Two previous studies (Nagase et al., 2002; Busscher et al., 2006) have reported *S. sciuri* as the
148 most prevalent CoNS in horses. This observation is in contrast with our findings since *S.*
149 *vitulinus* was the frequently isolated from horses at all three equine facilities. This discrepancy
150 could be due to methodological factors. It should be noted that *S. vitulinus* and *S. sciuri* are
151 members of the *S. sciuri* group and discrimination between the two species is difficult by
152 conventional phenotypic methods (Stepanovic et al., 2004) such as those used in the two
153 earlier studies (Nagase et al., 2002; Busscher et al., 2006). The high frequency of *S. vitulinus*

154 detected in this study by 16S rDNA sequencing suggests that isolates belonging to this species
155 could have been misidentified as *S. sciuri* in the previous studies.

156

157 Historically, the presence of CoNS in clinical specimens is thought to be a result of
158 contamination in both human and veterinary medicine. During the last decade, some species,
159 mainly *S. epidermidis* and *S. haemolyticus*, have gained increasing attention as nosocomial
160 pathogens in humans. Although no information is available on the frequency of nosocomial
161 pathogens in veterinary hospitals, postoperative infections are a common complication in
162 equine surgery and can result in intensive postoperative care, prolonged hospitalization,
163 increased costs and mortality (Santschi, 2006). Schnellmann et al. (2006) showed that horses
164 entering a hospital become readily colonised by CoNS after hospitalisation. Persistence and
165 clonal spread of methicillin-resistant *S. haemolyticus* have been demonstrated within a
166 veterinary teaching hospital in Norway (Sidhu et al., 2007). CoNS are able survive on many
167 hospital surfaces such as floors, tables, fabric, gloves and medical devices (Neely et al., 2000).
168 The property of CoNS to adapt to veterinary hospital environments was confirmed by our
169 study. Both veterinary hospitals were found to be widely contaminated with CoNS, including
170 surgical and medical equipment (Table 1).

171

172 Our study illustrates that MRCoNS clones, including clinically-relevant species such as *S.*
173 *haemolyticus*, can be shared by and possibly exchanged between horses and humans in close
174 contact, and that the environment of veterinary hospitals can be widely contaminated with
175 these bacteria. Although human and animal health risks associated with these findings remain
176 unknown, CoNS human infections are usually caused by the patient's own colonizing strain
177 (Frebourg et al. 1999 and Costa et al. 2004). The study also provides interesting information

178 on ecology and epidemiology of MRCoNS within veterinary hospitals, and underlines the
179 importance of infection prevention and control best practices in equine medicine.

180

181 **Acknowledgments**

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184 sampling and isolation.

185

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263 coagulase-negative staphylococci. *J. Clin. Microbiol.* 42, 4947-4955.
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265 **Tables**

266 **Table 1:** Occurrence and species distribution of methicillin-resistant coagulase-negative
267 staphylococci (MRCoNS) among horses, humans and environmental sites at the three equine
268 facilities under study.

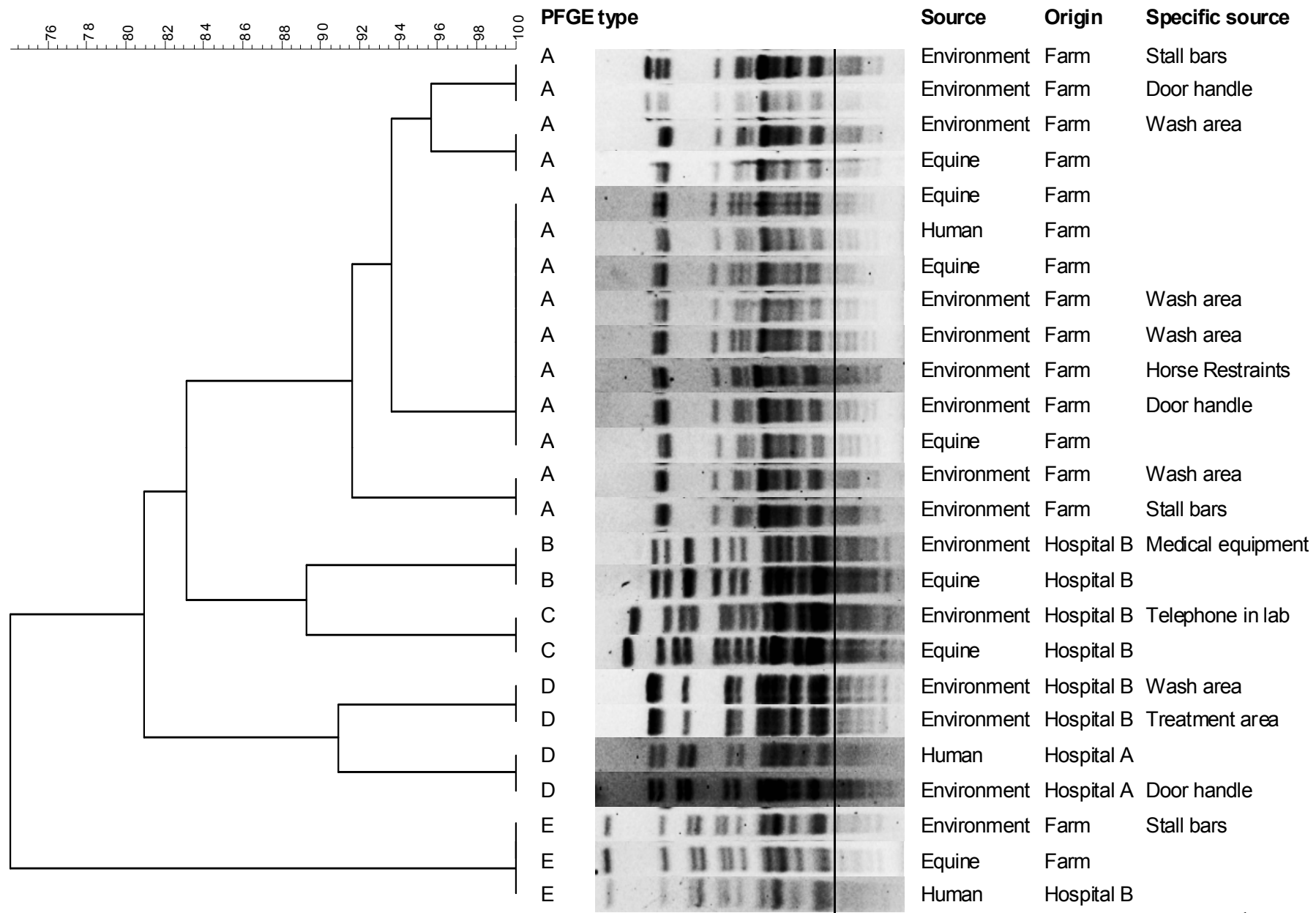
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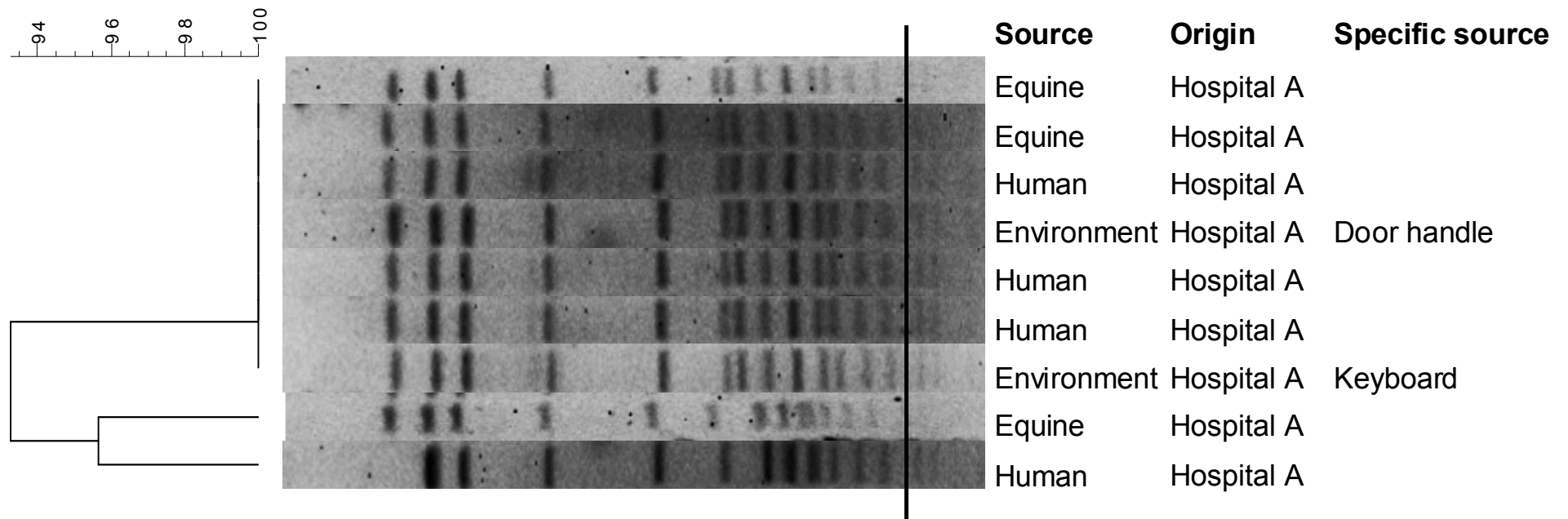
270 **Figure legend**

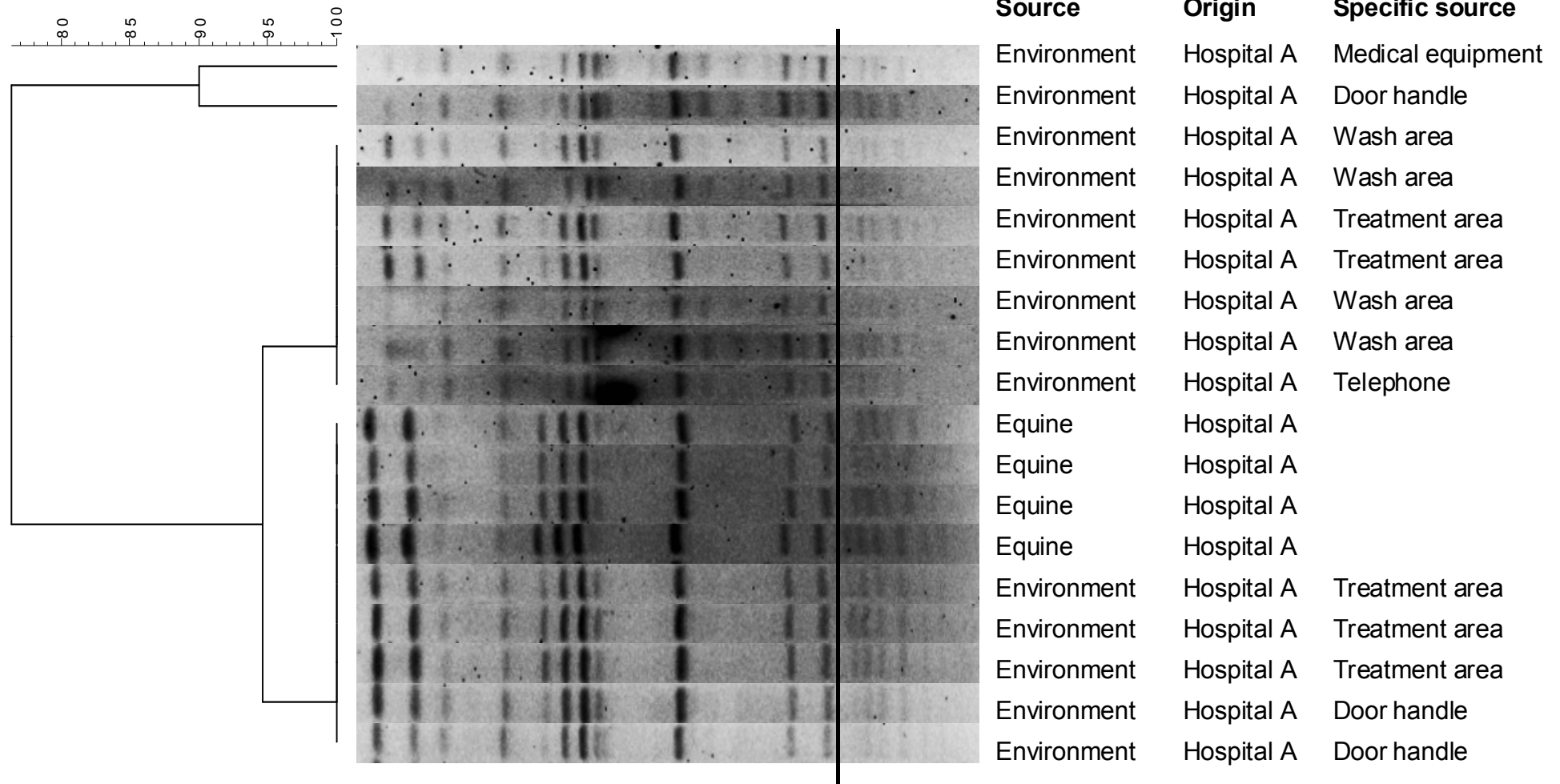
271 **Figure 1:** PFGE dendrogram of indistinguishable or closely-related equine, human and
272 environmental methicillin-resistant staphylococci identified as *S. vitulinus* (A), *S.*
273 *haemolyticus* (B) and *S. sciuri* (C). PFGE types were defined based on a 90% similarity cut-
274 off. Bands included in the cluster analysis ranged from 48.5 kb- 679kb. The line indicates the
275 lower limit of bands used in the cluster analysis.

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1a





1c

MRCoNS species	Horses (n=39)	Human (n=32)	Wash area (n=20)	Door handle (n=16)	Stalls or restraint (n=14)	Surgical and medical equipment (n=11)	Keyboard or phone (n=7)	Treatment area (n=7)	Radio (n=1)	Total
<i>S. epidermidis</i>	-	2	-	-	-	-	-	-	-	2
<i>S. equorum</i>	1	1	-	-	1	-	-	-	1	4
<i>S. haemolyticus</i>	3	6	3	1	-	-	1	-	-	14
<i>S. saprophyticus</i>	-	1	0	-	-	-	-	-	-	1
<i>S. sciuri</i>	10	1	4	3	-	1	1	5	-	25
<i>S. vitulinus</i>	18	9	8	7	8	3	1	2		56
Numbers of positive samples (%)	32 (82%)	20 (63%)	15 (75%)	11 (69%)	9 (64%)	4 (36%)	3 (43%)	7 (100%)	1 (100%)	102 (69%)