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1 **Prevalence of Methicillin-resistant *Staphylococcus pseudintermedius* isolated from**
2 **clinical samples of companion animals and equidae**

3
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27 1. Abstract

28 In this study the prevalence of Methicillin-resistant *Staphylococcus pseudintermedius*
29 (MRSP) in clinical samples of different animal species was defined by investigating a total of
30 16,103 clinical samples originating from veterinary facilities of five German federal states in
31 2007. Of all samples examined, 72 were positive for MRSP, giving an overall prevalence of
32 0.45%. In clinical specimens originating from small animals the prevalence was 0.58% (n=67;
33 dogs n=61 and cats n=6), while samples from equidae revealed a prevalence of 0.10% (n=5;
34 horses n=4; donkey n=1). Forty-six representative phenotypically identified MRSP were
35 further differentiated by DNA-based species assignment, PCR detection of *mecA*, *SCCmec*-
36 typing and MIC-determination. As expected, all 46 isolates were unambiguously proven to be
37 MRSP by sequencing of housekeeping genes *pta* and *cpn60* and being positive for *mecA*.
38 Furthermore, all isolates harboured the mobile staphylococcal cassette chromosome *mec*
39 (*SCCmec*) *SCCmecIII*. Antibiotic susceptibility testing for 20 different conventional
40 antimicrobial agents disclosed a high rate of multidrug-resistant isolates (45 of 46) displaying
41 an identical or at least similar resistance pattern for non- β -lactam antimicrobials. The
42 recognised prevalence of MRSP, which have already been shown to be potential zoonotic
43 agents, reflects the recently emerging development of these serious and often multidrug-
44 resistant pathogens in Germany.

45

46 2. Introduction

47 Coagulase-positive Staphylococci (CPS), especially members of the
48 *Staphylococcus intermedius*-group (*S. intermedius*, *S. pseudintermedius*, *S. delphini*), are
49 opportunistic pathogens in various animal species, particularly in dogs and cats (Biberstein et
50 al., 1984; Cox et al., 1988). Conventional microbiological diagnostic tests often fail to
51 distinguish between *S. pseudintermedius* and *S. intermedius*, leading to the assumption, that
52 *S. pseudintermedius* were frequently misidentified as *S. intermedius* or *S. aureus* (Sasaki et

53 al., 2007b; Van Hoovels et al., 2006). Recently, *S. pseudintermedius* was found to act as an
54 important pathogen of skin and soft tissue infections in pet animals, especially as a major
55 cause of canine pyoderma (Bannoehr et al., 2007). Being known only as a distinct species
56 since 2005 (Devriese et al., 2005), many studies concerning methicillin-resistance in
57 coagulase-positive staphylococci did not include the species *S. pseudintermedius* (Griffeth et
58 al., 2008; Morris et al., 2006). Consequently, knowledge concerning the prevalence,
59 particularly of SCC*mec*-types of methicillin-resistant *S. pseudintermedius* (MRSP) is scarce.
60 Since 2002, we routinely screen CPS isolated from clinical samples for *mecA* by PCR. In
61 2006, a sudden rise in isolation of Methicillin-resistant SIG (MRSIG) from clinical specimens
62 of animal origin was recognized. Due to limited data available on the occurrence, prevalence
63 and antimicrobial resistance pattern of methicillin-resistant *S. pseudintermedius* (MRSP), in
64 2007 we conducted a prevalence study, including genotypic species verification, SCC*mec*-
65 typing and MIC determination. To increase the epidemiological validity, we collaborated with
66 a larger diagnostic laboratory, thereby enlarging the sample size.

67

68 **3. Materials and Methods**

69 **3.1 Sampling**

70 A total of 16,103 clinical specimens derived from diseased animal patients in different
71 veterinary hospitals and smaller facilities were investigated during 2007. All specimens were
72 sent in for microbiological diagnostic testing of to the diagnostic section of the Institute of
73 Microbiology and Epizootics (IMT Berlin) and Synlab-vet (Labor Augsburg).

74 **3.2 Bacterial isolation and MRS confirmation**

75 **Bacterial isolation**

76 All diagnostic specimens were routinely streaked onto the following media: Standard nutrient
77 agar I (Roth GmbH, Karlsruhe, Germany) charged with 5% defibrinated sheep blood, Chrom
78 agar orientation (Mast Diagnostica, Reinfeld, Germany) and Gassner agar (Sifin, Berlin,

79 Germany). Hemolysis and growth characteristics were evaluated after aerobic incubation at
80 37°C, first after 18h and a second time after 36h. Presumptive SIG were identified based on
81 colony morphology, hemolysis, Gram stain appearance, catalase test, tube coagulase reaction
82 and the ability to produce acid from different carbohydrates according to (Bannermann and
83 Peacock, 1999) or by employing the automated BD Phoenix System (Becton Dickinson,
84 Diagnostic Systems, Sparks, MD, USA) in accordance with manufacturer's instructions.
85 Phenotypic testing for differentiation between isolates within the *Staphylococcus intermedius*-
86 group was completed by detection of ADH (Arginine Dihydrolase, DIATABS, Rosco
87 Diagnostica, Taastrup, Denmark)- and DNase (DNase-agar, Oxoid, Wesel, Germany) activity.
88 Screening for methicillin-resistance in isolates belonging to the SIG was initially performed
89 by the disk diffusion method using Oxacillin (5µg) and Cefoxitin (30µg) as recommended by
90 the CLSI, 2004 on Mueller-Hinton agar plates (Roth GmbH, Karlsruhe, Germany). Samples
91 were incubated at 35°C for 24h. For Oxacillin, breakpoints were those recommended for
92 bacterial isolates from animals (CLSI, 2004) and for Cefoxitin, we used breakpoints as
93 recommended for human CPS isolates (CLSI, 2005).

94 **MRS confirmation**

95 Verification of Methicillin-resistance in SIG and distinct exclusion of *S. aureus* was
96 performed by PCR according to Merlino et al. (Merlino et al., 2002).

97 ***S. pseudintermedius* species verification**

98 DNA-sequence analysis of internal fragments of two conserved housekeeping genes (*cpn60*,
99 *pta*) was used for species verification (Bannoehr et al., 2007). Analysis was carried out on a
100 representative selection of 46 *S. pseudintermedius* isolates, including all isolates of cats (n=6),
101 horses (n=4), the donkey and further 35 randomly chosen isolates from dogs. PCR-products
102 were purified and DNA-sequence-analyzed by Agowa-Sequence-Service (Agowa, Berlin,
103 Germany). Alignment of the sequences was performed with deposited *pta* and *cpn60* -
104 sequences of *S. pseudintermedius* N940276 and HH4 (GenBank accession numbers

105 EU157608 and EU157503), *S. intermedius* NCTC 11048 (GenBank accession number
106 EU157414) and *S. delphini* ATCC 49171 (GenBank accession number EU 157507) at NCBI
107 GenBank (www.ncbi.nlm.nih.gov).

108 **3.3 SCCmec-Typing**

109 Primer sets and PCR-conditions for SCCmec-Typing, were used as described previously
110 (Boyle-Vavra et al., 2005; Hanssen et al., 2004; Ito et al., 2004). Detection of pT181 was
111 conducted according to Ito et al, 2001. As reference strains for PCR-determination of *ccr*- and
112 *mec*-elements, NCTC 10442, N315, WIS, 85/2082 and CECT 231 were used.

113 **3.4 Antimicrobial susceptibility testing**

114 The broth microdilution method with automated BD Phoenix was used for MIC determination
115 of 20 antimicrobial agents according to CLSI guidelines and clinical breakpoints (Clinical
116 And Laboratory Standards Institute, 2004) for the representative 46 MRSP isolates. The tested
117 panel is listed in table3.

118 **3.5 Statistical analysis**

119 Significance was tested with chi-square test (χ^2) and by difference in proportion of two
120 independent samples for the MRSP distribution among body sites of infection as well as for
121 the distribution of MRSI in samples from different animal species.

122

123 **4. Results**

124 **Prevalence**

125 Altogether 16,103 clinical specimens were examined in two diagnostic veterinary laboratories
126 in 2007 for the occurrence of MRSP. Of these, a total of 870 isolates belonging to the SIG
127 were identified (see: table 2) and the percentage of SIG within all coagulase-positive
128 staphylococci amounted to 76.2%. The overall prevalence of MRSP within all clinical
129 specimens sent in for diagnostic purposes from dogs, cats and equidae was 0.45% (n=72).
130 Within all CPS, MRSP represented 6.3% and MRSA 2.5% (for more detailed data: see table

131 2). The prevalence of MRSP in specimens from small animal origin was amounted to 0.58%,
132 i.e. in dogs 0.8% (61 out of 7,490) and 0.1% in cats (6 out of 3,903). A total of five MRSP
133 were isolated from specimens of equid origin (horses n=4; donkey n=1), thus the prevalence
134 of MRSP in specimens obtained from horses and donkeys was 0.1% (5 out of 4710).
135 Comparing prevalence rates among the different animal species, MRSP prevalence in
136 specimens from dogs was significantly higher than in cats and equidae ($p < 0.001$). Regarding
137 specimens from all animal species investigated, most MRSP (40.3%; n=29) were isolated
138 from wound infections followed by specimens from the auditory channel (33.3%; n=24).
139 Specimens from skin and mucosa and the urinary tract amounted to 8.3% (n=6). Additional
140 details regarding the distribution of isolated MRSP among single animal species and body
141 sites of specimen origin are given in table 1.

142 **Species verification and SCCmec-typing**

143 Out of 72 MRSP identified, 46 representative MRSP isolates were further characterized by
144 molecular typing. Genes *pta* and *cpn60* of all strains were a hundred percent identical to the
145 deposited sequences of the *S. pseudintermedius* strains N940276 and HH4, respectively.
146 DNA-sequence similarity with *S. intermedius* strain NCTC11048 and *S. delphini* ATCC
147 49171 for *pta* and *cpn60* varied between 92% and 97%. Furthermore, all 46 MRSP harboured
148 SCCmecIII, by distinct detection of *ccrA3*, *ccrB3*, *mecI*, *mecRA*, *mecRB* and pT181.

149 **Antimicrobial susceptibility testing**

150 Out of 46 representative isolates, 45 (97.8%) showed multiple resistances in non- β -lactam
151 antimicrobials including erythromycin, clindamycin, gentamicin, tobramycin, ciprofloxacin,
152 levofloxacin, and trimethoprim/sulfamethoxazol. While MIC results for some β -lactam
153 antimicrobials showed variable results, all 46 MRSP showed unambiguous resistance to
154 Oxacillin (table 3).

155

156

157 **5. Discussion**

158 Although MRSP were earlier known to be an infective causative agent for animals (Bannoehr
159 et al., 2007), only little is known about their particular importance in veterinary medicine in
160 general. In this study, MRSP were identified in diagnostic material derived from clinically
161 diseased dogs, cats, horses and one donkey. The reported rate of 6.3% MRSP within all CPS
162 in contrast to 2.5% for MRSA substantiates the clinical relevance of MRSP for veterinarians
163 as well as for veterinary microbiological laboratories. Nevertheless, we have to consider some
164 limitations regarding prevalence rates due to the inability to discriminate infection from
165 colonization or contamination in each case, which is inevitable to some degree. Similar to
166 former results, no MRSI positive isolates were found during this study, leading to the
167 assumption that earlier studies concerning MRSI should probably apply to MRSP (Hanselman
168 et al., 2008; Sasaki et al., 2007b).

169 In this study, the rate of MRSP isolated from specimens of wounds was significantly higher
170 than those from all other infection sites ($p < 0.001$). Thus, MRSP have turned out to be an
171 important pathogen of wound infections in small animals, particularly in dogs, and are
172 occasionally even associated with wound infections in horses

173 The discriminating power of DNA-sequence analysis of internal fragments of *cpn60* and *pta*
174 for differentiation of SIG has only recently been shown (Bannoehr et al., 2007; Sasaki et al.,
175 2007b). In this study, genotypic species verification by DNA-sequence analysis of these two
176 highly conserved housekeeping genes showed 100% accordance with phenotypic typing
177 results including DNase and argininedihydrolase-testing and allowed verification of 46
178 representative isolates as *S. pseudintermedius*. This finding has led us to recommend a
179 combination of testing for enzymatic activity of argininedihydrolase and DNase as a useful
180 diagnostic tool to differentiate staphylococci of the *intermedius* –group.

181 Interestingly, SCC*mec*-typing results in this study only revealed one SCC*mec*-type, namely
182 SCC*mec*III, among the 46 MRSP further investigated. While SCC*mec*V, several non-typeable

183 *SCCmec*-types and new *SCCmec* have been reported in strains from the USA, Japan and
184 Switzerland, *SCCmecIII* seems to be more frequently associated with MRSP in Germany
185 (Descoux et al., 2008; Moodley et al., 2008; Sasaki et al., 2007a). So far, the origin of
186 *SCCmec* in MRSP is unknown, but horizontal gene transfer of *SCCmec* between different
187 species of staphylococci has already been previously assumed (Hanssen et al., 2004). Animals
188 colonized with *mecA*-positive coagulase-negative staphylococci (Vengust et al., 2006) or
189 MRSA (Walther et al., 2008) could thereby serve as a potential source for *SCCmec* in SIG.
190 Even transmission of *SCCmec* between MRSP and hitherto Methicillin susceptible
191 staphylococci, for example *S.aureus*, must be considered. Nevertheless, further information
192 concerning acquisition and transmission routes of *SCCmec* is urgently needed.

193 In our study, antimicrobial susceptibility testing revealed a remarkable multidrug resistance
194 towards the majority of non- β -lactam antimicrobials like fluorquinolones, aminoglycosides,
195 and macrolides in 45 of 46 investigated MRSP, showing a similar resistance pattern for all 45
196 isolates. MIC results for different β -lactam antimicrobials differ widely among the MRSP,
197 except those for Oxacillin, which presented a resistant result for all strains investigated here.
198 Especially for Cefoxitin MICs of MRSP, similar ambiguous results have been recently
199 reported (Bemis et al., 2008). Therefore, we recommend an Oxacillin-based approach for
200 phenotypical identification of MRSP.

201 SIG are common components of the skin, oral and nasal flora of several mammalian species
202 (Biberstein et al., 1984) and increasing prevalence of MRSP in companion animals and horses
203 reported from different countries probably reflects a minatory development. While human
204 infections due to SIG were only reported sporadically (Campanile et al., 2007), a first case of
205 human MRSP-infection was recently published (Van Hoovels et al., 2006). Animal to human
206 transmission of methicillin-resistant staphylococci or vice versa, as it has been assumed for
207 MRSA (Weese et al., 2006), should be contemplated as a potential problem. In conclusion,
208 further epidemiological studies concerning MRSP in veterinary settings and the community

209 are needed, including detailed clinical data, analysis of potential transmission pathways in
210 addition to comprehensive microbiological typing approaches.

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Table 1: Distribution of animal species and body site of infection of 72 isolated

Animal species	Body site of infection																
	<i>n</i>	Wounds (%)	Urogenital-tract (%)	Auditory channel (%)	Skin and mucosa (%)	Gastrointestinaltract (%)	Eye and conjunctiva (%)	Systemic infection (%)	Other sites*	(%)	(%)	(%)	(%)	(%)	(%)	(%)	
all species	72	29	40.3	6	8.3	24	33.3	6	8.3	2	2.8	1	1.4	3	4.2	1	1.4
Dog	61	24	39.3	3	4.9	23	37.7	6	9.9	0	0	1	1.6	3	4.9	1	1.6
Cat	6	2	33.3	3	50	1	16.7	0	0	0	0	0	0	0	0	0	0
Horse	4	3	75	0	0	0	0	0	0	1	25	0	0	0	0	0	0
Donkey	1	0	0	0	0	0	0	0	0	1	100	0	0	0	0	0	0

Abbreviations: *n*, number; * joint aspirate

1 Table 2: Distribution of Coagulase-positive staphylococci among isolates of small animals and equidae in 2007

Animal species	<i>n</i>	Coagulase-positive Staphylococci (CPS)*											
		SIG ¹		<i>S. aureus</i>		MRSP		MSSIG ²		MRSA		MSSA	
		(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
total	1141	870	76.2	271	23.8	72	6.3	798	70.0	29	2.5	242	21.2
Dog	901	821	91.1	80	8.9	61	6.8	760	84.3	15	1.7	65	7.2
Cat	91	26	28.6	65	71.4	6	6.6	20	22.0	11	12.1	54	59.3
Horse	148	22	14.9	126	85.1	4	2.7	18	12.2	3	2.0	123	83.1
Donkey	1	1	100	0	0	1	100	0	0	0	0	0	0

*except *S. hyicus*

¹Staphylococci of the intermedius-group: *S. intermedius*, *S. delphini*, *S. pseudintermedius*; ² other *mecA*-negative SIGs, species not closer identified

Abbreviations:

n, number; MRSP, Methicillin-resistant *S. pseudintermedius*; MSSIG, Methicillin-susceptible SIGs; MRSA, Methicillin-resistant *S. aureus*; MSSA, Methicillin-susceptible *S. aureus*

2
3

Table 3: Results of MIC-Determination of 20 antimicrobial agents for 46 MRSP

Antimicrobial agent*	MIC Range, $\mu\text{g/ml}$	resistant (%)	intermediate (%)	susceptible (%)			
non-β-lactam antimicrobials							
Clindamycin	$\leq 0.5 - > 2$	45	97.8	0	0	1	2.2
Ciprofloxacin	1 - > 2	45	97.8	0	0	1	2.2
Erythromycin	$\leq 0.25 - > 4$	45	97.8	0	0	1	2.2
Fosfomycin	$\leq 16 - 64$	0	0	45	97.8	1	2.2
Fusidic acid	≤ 4	0	0	0	0	46	100
Gentamicin	> 8	46	100	0	0	0	0
Levofloxacin	$\leq 1 - > 4$	45	97.8	0	0	1	2.2
Linezolid	$\leq 1 - 4$	0	0	0	0	46	100
Moxifloxacin	$\leq 1 - > 4$	45	97.8	0	0	1	2.2
Rifampin	≤ 0.5	0	0	0	0	46	100
Teicoplanin	≤ 1	0	0	0	0	46	100
Tetracyclin	$\leq 0.5 - > 8$	28	60.9	3	6.5	15	32.6
Tobramycin	8 - > 8	44	95.7	2	4.3	0	0
Trimethoprim/Sulfamethoxazol	$> 2 / 38$	46	100	0	0	0	0
Vancomycin	$\leq 1 - 4$	0	0	0	0	46	100
β-lactam antimicrobials							
Ampicillin/Sulbactam	$\leq 4/2 - > 16/8$	37	80.4	NA	0	9	19.6
Cefoxitin	$\leq 2 - > 16$	12	26.1	0	0	34	73.9
Imipenem	$\leq 2 - 8$	0	0	1	2.2	45	97.8
Meropenem	$\leq 2 - 8$	13	28.3	2	4.3	31	67.4
Oxacillin	> 2	46	100	0	0	0	0

* Breakpoints according to Clinical And Laboratory Standards Institute (CLSI), approved standard M100-S15, M31-S1
Abbreviations: NA, no CLSI-approved breakpoint available