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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- $\beta$ -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 ( $\chi^2$ ) 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 SCC*mec*-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 SCC*mec*III, 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- $\beta$ -lactam  
151 antimicrobials including erythromycin, clindamycin, gentamicin, tobramycin, ciprofloxacin,  
152 levofloxacin, and trimethoprim/sulfamethoxazol. While MIC results for some  $\beta$ -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 SCC*mec*-types and new SCC*mec* have been reported in strains from the USA, Japan and  
184 Switzerland, SCC*mec*III 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 SCC*mec* in MRSP is unknown, but horizontal gene transfer of SCC*mec* 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 SCC*mec* in SIG.  
190 Even transmission of SCC*mec* 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 SCC*mec* is urgently needed.

193 In our study, antimicrobial susceptibility testing revealed a remarkable multidrug resistance  
194 towards the majority of non- $\beta$ -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  $\beta$ -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*	(%)	(%)	(%)	(%)	(%)	(%)	(%)	
<b>all species</b>	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
<b>Dog</b>	61	24	39.3	3	4.9	23	37.7	6	9.9	0	0	1	1.6	3	4.9	1	1.6
<b>Cat</b>	6	2	33.3	3	50	1	16.7	0	0	0	0	0	0	0	0	0	0
<b>Horse</b>	4	3	75	0	0	0	0	0	0	1	25	0	0	0	0	0	0
<b>Donkey</b>	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 <sup>1</sup>		<i>S. aureus</i>		MRSP		MSSIG <sup>2</sup>		MRSA		MSSA	
		(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
<b>total</b>	<b>1141</b>	<b>870</b>	<b>76.2</b>	<b>271</b>	<b>23.8</b>	<b>72</b>	<b>6.3</b>	<b>798</b>	<b>70.0</b>	<b>29</b>	<b>2.5</b>	<b>242</b>	<b>21.2</b>
<b>Dog</b>	901	821	91.1	80	8.9	61	6.8	760	84.3	15	1.7	65	7.2
<b>Cat</b>	91	26	28.6	65	71.4	6	6.6	20	22.0	11	12.1	54	59.3
<b>Horse</b>	148	22	14.9	126	85.1	4	2.7	18	12.2	3	2.0	123	83.1
<b>Donkey</b>	1	1	100	0	0	1	100	0	0	0	0	0	0

\*except *S. hyicus*

<sup>1</sup>Staphylococci of the intermedius-group: *S. intermedius*, *S. delphini*, *S. pseudintermedius*; <sup>2</sup> 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 (%)			
<b>non-<math>\beta</math>-lactam antimicrobials</b>							
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	$\leq 4$	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	$\leq 0.5$	0	0	0	0	46	100
Teicoplanin	$\leq 1$	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
<b><math>\beta</math>-lactam antimicrobials</b>							
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