Pregnancy and conception rate after two intravaginal inseminations with dog semen frozen either with 5% glycerol or 5% ethylene glycol

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Isolation of methicillin-resistant *Staphylococcus pseudintermedius* from breeding dogs

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Abstract

The overuse of antimicrobials can select resistant bacteria strains; staphylococci have the ability to become resistant to all beta-lactam antimicrobials and are a significant concern in human medicine and a growing issue for veterinary medicine. Since in breeding kennels antimicrobials are sometimes incorrectly used, the objective of the work was to assess the occurrence of methicillin-resistant coagulase-positive staphylococci in breeding dogs. The research was carried out in 13 kennels that were allotted to three categories according to the intensity of antimicrobial use. Vaginal and milk swabs were taken from 87 healthy bitches around parturition and also from multiple organs of 27 of their pups that died within the first 2 weeks. Standard bacteriological examinations were carried out and coagulase-positive staphylococci were identified. All the coagulase-positive staphylococci resulted to be *Staphylococcus pseudintermedius*. Susceptibility to oxacillin and the presence of the *mecA* gene were tested. Nine out of 89 strains (six isolated from the bitches' milk and three from dead puppies, all belonging to kennels characterized by an excessive use of antimicrobials) were multidrug-resistant, methicillin-resistant and *mecA* positive.

Our results confirm that excessive use of antimicrobials entails the risk of selecting resistant staphylococci strains. Our data also indicate that the bacterial flora of healthy dogs belonging to specific populations may act as a reservoir of resistance genes.

Keywords: *Staphylococcus pseudintermedius*; methicillin-resistance; *mecA* gene; dog

1. Introduction

Coagulase-positive staphylococci, (*Staphylococcus aureus, S. intermedius, S. pseudintermedius* and *S. delphini*) are commensal bacteria and opportunistic pathogens in most animal species [1]. Resistance to methicillin appeared in *S. aureus* in 1960 [2] and originated because of widespread use of beta-lactam antibiotics in nosocomial settings; the frequency of infections due to methicillin-resistant *S. aureus* has steadily increased over the years, representing a serious problem in human medicine [3]. Resistance to methicillin is due to the presence of the *mecA* gene, which encodes the altered penicillin-binding protein 2a (PBP2a) that has a reduced affinity for all beta-lactam antimicrobials; the *mecA* gene is carried on a mobile DNA element, the staphylococcal cassette chromosome *mec* (SCC*mec*) [4].

The most prevalent coagulase-positive staphylococcal species in dogs, isolated both from normal skin and oronasal mucosa and from skin and ear infections [5,6], was *S. intermedius.* Recently, using a molecular identification approach, canine isolates that had previously been phenotipically identified as *S. intermedius* were reclassified as *S. pseudintermedius* [7]. Devriese et al. [8] proposed that canine strains identified by traditional means should be reported as *S. pseudintermedius* unless shown by genomic investigation to belong to other related species. A small proportion of *S. intermedius* (old classification) strains isolated both from healthy dogs [9,10] and from dogs showing skin and ear infections [6] has proven to be methicillin-resistant *mecA* positive. Although *S. intermedius* (old classification) is seldom responsible of human infections, transfer from dog to human is possible [11,12].

Since antimicrobials are sometimes misused in breeding kennels, the aims of this work were 1) to assess the occurrence of methicillin-resistant coagulase-positive

staphylococci in breeding dogs; and 2) to compare the frequency of isolation of resistant strains among kennels differing in the use of antimicrobials.

2. Materials and Methods

2.1 Animals and sampling

Eighty-seven breeding bitches housed in 13 kennels located in Northern Italy were included in the study. The history of the kennels reported a different use/misuse of antibiotics, in particular around parturition, and they were consequently divided into three categories: 1) administration of amoxicillin or amoxicillin-clavulanic acid only when required by pathological conditions (5 kennels, 36 bitches); 2) almost routine administration of amoxicillin or amoxicillin-clavulanic acid around parturition (5 kennels, 29 bitches); 3) routine administration of various antimicrobial agents around parturition (II and III generation cephalosporins, macrolids, adopted after amoxicillin and amoxicillin-clavulanic acid had become ineffective) (3 kennels, 22 bitches).

Vaginal swabs were taken from the bitches 7-10 days before parturition and milk samples were collected 7-10 days after parturition; the bitches belonging to kennels of category II and III were under antimicrobial treatment when milk cultures were carried out. A guarded sterile swab (Copan Innovation®, Brescia, Italy) was introduced into the cranial vagina after thorough disinfection of the vulva with a povidone-iodine solution, and placed in the Amies transport medium provided with the swab. A drop of milk was collected on a sterile swab (Copan Innovation®, Brescia, Italy) from each of the caudal mammary glands, after local disinfection. A complete post-mortem examination was performed, including culture (from brain, stomach, intestine, liver, heart, lungs) and sensitivity testing, on puppies that died within the first 2 weeks. All the bitches, aged 1.5-9 years and belonging to different breeds, were in good health at the moment of

sampling and no signs or symptoms of either mastitis or metritis were present during the period of observation.

2.2 Isolation of coagulase-positive Staphylococci

Swabs were inoculated onto Blood Agar Base n°2 (Oxoid, Basingstoke, United Kingdom) with 5% defibrinated sheep blood (DiaTech, Jesi, Italy) and streaked out to obtain single colonies. After 24 hrs of incubation at 37±1 °C in aerobic conditions, presumptive identification of staphylococci was based on colony morphology, Gram stain appearance, catalase test, haemolysis, pigment production. Coagulase-positive staphylococci were identified by coagulase reaction on rabbit plasma (Istituto Zooprofilattico delle Venezie, Legnaro, Italy) and clumping factor by Slidex Staph (BioMérieux, Marcy l'Etoile, France). API Staph ID 32 phenotypic test (BioMérieux, Marcy l'Etoile, France) and a specific PCR analysis for *S. aureus* [13] were also carried out.

2.3 Identification of S. pseudintermedius

S. pseudintermedius identification was performed according to Bannoehr et al. [14]. One colony of each isolate was suspended in 1 ml Phosphate Buffered Saline (pH 7.4) and DNA was extracted using DNeasy Blood & Tissue Kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions for Gram positive bacteria. The amplification was performed in a total volume of 50 µl containing 5 µl of DNA, 1.5 mM of $MgCl_2$, 0.1 U/µl of Fast Start Taq DNA Polymerase (Roche Diagnostics, Mannheim, Germany), 200 µM of each dNTPs (Applied Biosystems, Foster City, USA) and 0.5 µM of primers pta_f1 and pta_r1 (Table 1) [14]. DNA amplification was carried out in an Eppendorf Mastercycler Ep Gradient S (Eppendorf, Milano, Italy) with the following thermal cycling conditions: initial denaturation at 95 °C for 4 min, followed

147 by 40 cycles of amplification, denaturation at 95 °C for 30 s, annealing at 55 °C for 30
148 s, extension at 72 °C for 60 s, ending with a final elongation step of 5 min at 72 °C.
149 After amplification, 10 µl of PCR products were subjected to enzymatic restriction with
150 1.5 U/µl of *Sau3AI* for 1h at 37 °C and the restriction products subsequently subjected
151 to agarose gel electrophoresis. Restriction of *S. pseudintermedius* amplicons resulted in
152 two fragment of 213 pb and 107 pb.
153
154 *2.4 Antimicrobial susceptibility*
155 Susceptibility to a panel of sixteen antimicrobial agents was determined by the disk
156 diffusion method in Mueller-Hinton agar (Sclavo Diagnostics International, Siracusa,
157 Italy) according to the guidelines of the Clinical Laboratory Standards Institute, when
158 available [15, 16].

159 Discs of penicillin G (10 IU), ampicillin (10 µg), amoxicillin-clavulanic acid (20+10
160 µg) gentamicin (10 µg), oxacillin (1 µg), cefalexin (30 µg), cefuroxime (30 µg),
161 spiramycin (100 µg), streptomycin (10 µg), tetracydine (30 µg), tilmicosin (15 µg),
162 tylosin (30 µg), enrofloxacin (5 µg), rifampin (5 µg), tiamulin (30 µg), trimethoprim-
163 sulfamethoxazole (1.25+23.75 µg) were tested. Interpretative criteria for the inhibition
164 zone diameters of tylosin, spiramycin and streptomycin were given by the
165 manufacturers.

166 Bacteria strains were stored at -80 °C and the susceptibility test was repeated after
167 species identification.

168
169 *2.5 Oxacillin resistance confirmation*
170 Coagulase-positive staphylococci strains were transferred onto a selective medium,
171 Oxacillin Resistance Screening Agar Base with selective supplement (Oxoid,

Basingstoke, United Kingdom) (oxacillin concentration: 2µg/ml), and incubated at 37°C±1°C for 24 h to confirm susceptibility to oxacillin, as a surrogate for methicillin.

2.6 MecA gene

Coagulase-positive staphylococci strains were tested for the presence of the *mecA* gene by PCR, using the primers reported in Table 1 [17]. DNA was extracted from each sample as previously described. The amplifications were performed in a total volume of 25 µl containing 5 µl of DNA, 3 mM $MgCl_2$, 0.05 U/µl FastStart Taq DNA Polymerase (Roche Diagnostics, Mannheim, Germany), 200 µM of each dNTPs (Applied Biosystems, Foster City, USA) and 0.5 µM of primer. DNA amplification was carried out in an Eppendorf Mastercycler Ep Gradient S (Eppendorf, Milano, Italy) with the following thermal cycling conditions: initial denaturation at 95 °C for 6 min, followed by 30 cycles of amplification, denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, ending with a final elongation step of 5 min at 72 °C. After amplification, 10 µl of PCR products were subjected to agarose gel electrophoresis.

2.7 Statistical analysis

The frequency of isolation of coagulase-positive staphylococci, both as a whole and in each of the two localisations, was compared among the three categories of breeding kennels by using chi-squared analysis. The frequency of isolation of methicillin-resistant coagulase-positive staphylococci strains was analyzed using the Fisher exact test, considering kennels of category 1 and 2 as a unique category. A P value < 0.05 was considered significant.

3. Results

198 A total number of 76 coagulase-positive staphylococci strains were isolated from the
199 bitches (33 from vagina and 43 from milk) and 13 coagulase-positive staphylococci
200 strains were isolated from 27 dead puppies.
201 All the coagulase-positive staphylococci resulted to be *S. pseudintermedius*.
202 The frequency of *S. pseudintermedius* colonization was not significantly different
203 among the three categories of kennels, neither as a whole nor in each of the two
204 localisations. In 7 out of 13 cases of dead puppies, *S. pseudintermedius* had caused
205 septicaemia: in six cases it was also the only bacterium isolated from the milk of the
206 corresponding dams, and in one case it was isolated from the dam's vagina.
207 All the isolated *S. pseudintermedius* strains showed high percentages of resistance to
208 different antimicrobials, particularly to penicillin and ampicillin, spiramycin and
209 tetracycline (Table 2).
210 Eight strains isolated from the bitches' milk and three from the dead pups were
211 resistant to oxacillin; seven of the bitch strains and all the pup strains confirmed the
212 resistance in the selective medium, with the higher oxacillin concentration. Six out of
213 seven strains isolated from the bitches and all three strains isolated from the dead pups
214 carried the *mecA* gene. In the *mecA* negative bitch strain, either *mecA* was not present
215 or mutations in the primer region may have occurred.
216 The origin, antibiotic resistance characteristics and presence/absence of the *mecA* gene
217 in the methicillin-resistant strains are reported in Table 3. All these strains show
218 multidrug resistance. All the isolates shown in Table 3 came from two kennels
219 belonging to category 3. The isolates with numbers 6 and 7 came from two bitches,
220 while those bearing numbers 8 and 9 derived from their dead puppies. Overall, the
221 prevalence of *mecA* positive methicillin-resistant strains was 7.9% in the bitches; the
222 percentage of *mecA* positive methicillin-resistant strains on all *S. pseudintermedius*
223 isolates was 10.1%.

Table 4 shows the number of methicillin-resistant *mecA* positive *S. pseudintermedius* strains in the three categories of kennels and in the two localizations of the bitches. The frequency of isolation of methicillin-resistant *mecA* positive *S. pseudintermedius* strains in the bitches was higher in kennels belonging to category 3 (χ^2 [Yates corrected]=12.48; $P<0.001$).

All the staphylococci strains that resulted susceptible to oxacillin by disk diffusion test confirmed their susceptibility in the selective medium and resulted *mecA* negative.

4. Discussion

Antimicrobials are sometimes misused in breeding kennels and different agents may be routinely administered around parturition to prevent neonatal infections, instead of adopting more appropriate measures to minimise structural predisposing factors [18].

By comparing the antibiotic resistance in the intestinal flora, De Graef et al. [19] found higher resistance percentages in dogs kept in breeding kennels than in privately owned animals; the same study described also a relation between the antimicrobials used and the resistances of bacteria, thus suggesting the possibility that resistance genes may be transferred among bacteria colonising group-housed dogs.

All the coagulase-positive staphylococci strains isolated from the bitches in our work were identified as *S. pseudintermedius* by molecular analysis, in agreement with recent taxonomy observations on canine isolates [1,7,8].

In our study, *S. pseudintermedius* showed high percentages of antimicrobial resistance: also when bitches were not exposed to antimicrobial treatment before sampling, as in kennels of category 1, mean resistance rates for penicillin, ampicillin and spiramycin were higher than 80%, for tetracycline higher than 25% and for streptomycin higher than 18%. In a recent work [20], the Authors found a widespread occurrence of resistance in *S. pseudintermedius* from skin and ear infections of dogs with no recent

history of antimicrobial treatment. The high genetic polymorphism of the isolates suggested the possible transfer of resistance genes among *S. pseudintermedius* strains as well as other bacteria in the dog population and it could be worth testing this hypothesis also in our experimental series.

The occurrence of methicillin-resistance in *S. pseudintermedius* isolated in two breeding kennels, both belonging to the group characterised by excessive antimicrobials use, confirms that antimicrobial overuse selects resistant strains. Administration of antimicrobial drugs, specifically β-lactams and fluoroquinolones, in the previous 90 days, resulted in a significant risk factor for methicillin-resistant *S. aureus* (MRSA) infection in dogs [21].

Methicillin-resistant *S. pseudintermedius* (MRSP) colonization of healthy dogs living in a confined environment raises a number of issues including a) possible horizontal transfer from dog to dog, and particularly between females and males; b) the pattern of colonization over time; c) health hazard for dogs within kennels; and d) potential health hazard for breeders. A recent study by Loeffler et al. [22] showed that MRSA did not transmit readily between apparently healthy dogs housed in shared kennels and that MRSA carriage was only transient in a regularly cleaned and disinfected environment. However, these observations should be confirmed for MRSP and, furthermore, any infection developed by MRSP-colonized animals, would probably be difficult to treat. In fact, MRSP isolates that are positive for the *mecA* gene are generally multiresistant [23], as in our case, although not necessarily more virulent than their methicillin-susceptible counterparts [24]. A longitudinal study might help assess the persistence of MRSP carriage and, consequently, the effective risk that a group-housed dog population may become a reservoir for resistance genes. However, if antimicrobial pressure is not reduced in the concerned kennels, other resistant strains are going to be selected.

276 *S. intermedius* (old classification) is not usually a human pathogen, although it is known
277 to cause invasive infection following dog-bites [25]; sporadic cases of bacteraemia in
278 patients who had undergone invasive procedures have been reported [26], one case of
279 otitis externa [27] and, recently, a case of *S. pseudintermedius* catheter-related
280 bacteraemia in a hemophilic child [28]. Transmission of *S. intermedius* (old
281 classification) from dogs to their owners can occur [11,12] and it has already been
282 described also for methicillin-resistant strains [29]. The occurrence of *S. intermedius*
283 (old classification) in the owners of dogs affected by deep pyoderma was significantly
284 higher than in the control group [12]. However a recent investigation showed that
285 MRSP nasal colonization of the owners of dogs affected by MRSP skin infection was
286 uncommon and transient [30]. Nasal carriage of MRSP was detected in one staff
287 member of a veterinary teaching hospital [31]: personnel of veterinary clinics should be
288 made aware of the risk of nosocomial transmission of MRSP.
289 Although MRSP strains are not alarming as zoonotic agents, they represent a serious
290 concern as a potential source of horizontal gene transfer to other staphylococci,
291 converting methicillin-susceptible *S. aureus* colonizing humans into MRSA through
292 transfer of the mobile SCC containing the *mecA* gene [24,32]. *S. aureus* is indeed
293 thought to have acquired resistance through horizontal gene transmission from
294 coagulase-negative *S. sciuri* [33].
295 The prevalence of MRSP found in our study is higher than the values previously
296 reported for healthy dogs (1.5% [10]; 2% [9]) and is similar to data (6.3%) reported in
297 clinical samples of diseased dogs [10,34]. However, in our work, the frequency is
298 biased in kennels where the excessive use of antimicrobials has increased the selection
299 of resistant strains.
300 Genetic characterization of our MRSP isolates would be of great epidemiological
301 interest: their belonging to the multiresistant clonal lineage widespread in several

European countries (including Italy [35]), would confirm the spreading of MRSP strains with relatively stable clones. However, the assessment of the genetical profile of the isolates was out of the scope of this research.

A previous retrospective investigation on the bacteriological status of canine milk and septicaemia of neonatal puppies [36] excluded *S. intermedius* (old classification) as a major cause of septicaemia in neonates because, although isolated from the milk of the dams, the bacterium was never found in the organs of the septicaemic puppies. This issue deserves further investigation. In our work, *S. pseudintermedius* was isolated from the organs of septicaemic puppies and it was also the only bacterium isolated from the milk of the corresponding dams or from the dams' vagina. Two of the milk and puppy isolates were methicillin-resistant *mecA* positive. Although the isolates were not typed, a link between the strains from dam and puppy is very likely.

Our results confirm that the misuse of antimicrobials may lead to serious consequences because resistant strains may become a health hazard in veterinary and human medicine; methicillin-resistant staphylococcal infections may become causes of morbidity and mortality in companion animals but domestic animals may also represent a reservoir of infection and a zoonotic source [1]. MRSP should not only be considered as a nosocomial pathogen in veterinary settings [35] but also as a potentially problematic commensal in particular dog populations.

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439 Table 1. Primers used to identify *Staphylococcus pseudintermedius* and to detect the presence of the *mecA* gene

440

441

Primers	**Sequence 5'-3'**	**Product size (bp)**	**Reference**
Pta_f1	AAA GAC AAA CTT TCA GGT AA	320	[14]
Pta_r1	GCA TAA ACA AGC ATT GTA CCG		
*mecA*_F	AAA ATC GAT GGT AAA GGT TGG C	533	[16]
*mecA*_R	AGT TCT GCA GTA CCG GAT TTG C		

442

443 Table 2. Percentages of resistance to different antimicrobials of *Staphylococcus pseudintermedius* strains isolated in two localisations from bitches

444 housed in three categories of kennels

Kennel category	**1**		**2**		**3**	
Origin[a]	**M**	**V**	**M**	**V**	**M**	**V**
N° of isolates	21	13	9	12	13	8
Penicillin	85.7	92.3	88.9	100	100	100
Ampicillin	80.9	92.3	88.9	100	100	100
Amoxycillin-clavulanic acid	0	0	0	0	53.8	12.5
Oxacillin	0	0	0	0	61.5	0
Cefalexin	4.8	0	0	0	38.5	0
Cefuroxime	4.8	0	0	0	38.5	0
Spiramycin	90.5	69.2	88.5	90.0	100	100
Rifampin	4.8	0	0	0	0	0
Tilmicosin	0	7.7	0	10	84.6	12.5
Tylosin	0	15.4	22.2	30.0	100	37.5
Tetracycline	23.8	30.8	44.4	60.0	84.6	62.5
Streptomycin	14.3	23.1	55.6	30.0	92.3	37.5
Gentamycin	0	0	0	0	38.5	0
Enrofloxacin	0	0	0	0	53.8	0
Trimethoprim-sulfamethoxazole	9.5	15.4	11.1	20.0	84.6	12.5
Tiamulin	4.8	0	11.1	0	0	0

445 [a]M, milk; V, vagina.

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448 Table 3. Antibiograms of methicillin-resistant *Staphylococcus pseudintermedius* isolates. Dams and corresponding puppies are linked with brackets.

Strain	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Origin[a]	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**P**	**P**	**P**
Kennel category	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
Penicillin	R	R	R	R	R	R	R	R	R	R
Ampicillin	R	R	R	R	R	R	R	R	R	R
Amoxycillin-clavulanic acid	R	R	R	R	R	R	R	R	R	R
Oxacillin	R	R	R	R	R	R	R	R	R	R
Cefalexin	R	I	R	S	R	R	R	R	R	R
Cefuroxime	R	R	R	S	R	R	R	R	R	R
Spiramycin	R	R	R	R	R	R	R	R	R	R
Rifampin	S	S	S	S	S	S	S	S	S	S
Tilmicosin	R	R	R	R	R	R	R	R	R	R
Tylosin	R	R	R	R	R	R	R	R	R	R
Tetracycline	R	R	S	I	R	R	R	R	R	R
Streptomycin	R	R	R	R	R	R	R	R	R	R
Gentamycin	R	I	R	I	R	I	R	R	R	R
Enrofloxacin	R	R	R	R	R	R	R	R	R	R
Trimethoprim-sulfamethoxazole	R	R	R	R	R	R	R	R	R	R
Tiamulin	S	S	S	S	S	S	S	S	S	S
mecA	+	+	+	+	-	+	+	+	+	+

449 [a]M, milk; P, dead pup. R, resistant; S, susceptible; I, intermediate

Table 4. *Staphylococcus pseudintermedius* colonisation of the vagina and milk of the bitches belonging to the three categories of kennels (1= low, correct use of antimicrobials ; 2= moderate; 3= excessive); occurrence of methicillin-resistant *S. pseudintermedius* (MRSP) strains.

Kennel category	**1**	**2**	**3**
N of bitches	36	29	22
Percentage and (N) of bitches colonised by *S. pseundintermedius* in vagina and/or milk	66.7 (24)	65.5 (19)	77.3 (17)
Percentage and (N) of vaginal isolates	36.1 (13)	41.4 (12)	36.4 (8)
Percentage and (N) of milk isolates	52.8 (19)	37.9 (11)	59.1 (13)
Total N of isolates	32	23	21
Percentage and (N) of MRSP on vaginal isolates	0 (0/13)	0 (0/12)	0 (0/8)
Percentage and (N) of MRSP on milk isolates	0 (0/19)	0 (0/11)	46.1 (6/13)

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