Investigation of the antibiotic resistance and biofilm formation of Staphylococcus pseudintermedius *strains isolated from canine pyoderma*

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Keywords
Antibiotic resistance,
Biofilm,
Electron microscopy,
Microtitre Plate test,
Staphylococcus pseudintermedius.

Summary
The aim of the present study was to investigate the antibiotic resistance and biofilm formation among a collection of 51 clinical isolates of *Staphylococcus pseudintermedius* collected from canine pyoderma. All isolates were tested for the susceptibility to a panel of 14 antimicrobial agents by the disk diffusion method in Müeller-Hinton agar. Oxacillin resistance was detected by subculture on oxacillin screening agar base. Biofilm formation was investigated by the Microtitre Plate test (MtP) and for some strains by transmission electron microscopy (TEM). Antibiotic resistance profiling demonstrated that 45/51 *S. pseudintermedius* isolates had a multi drug resistant (MDR) phenotype, exhibiting simultaneous resistance to at least 3 antibiotics categories; whereas 6 isolates showed a non-MDR phenotype. Thirty strains (59%) were resistant in oxacillin resistant screening agar, the same strains were also positive for mecA by PCR assay. All *S. pseudintermedius* isolates showed biofilm production by MtP method. Seventeen out of 51 isolates were classified as weakly adherent, 26 as moderately adherent, and 8 as strongly adherent. Moreover, no difference in biofilm formation between meticillin-resistant *S. pseudintermedius* (MRSP) and meticillin-suscebtible *S. pseudintermedius* (MSSP) (*P* value > 0.05) was noted. The antimicrobial resistance mechanisms and biofilm formation could explain the difficulty in treating *S. pseudintermedius* canine infections, chemotherapeutic failure, and consequently persistent infections.

Indagine su antibiotico-resistenza e formazione di biofilm in ceppi di Staphylococcus pseudintermedius *isolati da piodermiti canine*

Parole chiave
Staphylococcus pseudintermedius,
Antibiotico-resistenza,
Biofilm,
Microscopia elettronica,
Test su piastre Microtitre.

Riassunto
Lo scopo del presente studio è stato quello di valutare l'antibiotico resistenza e la produzione di biofilm in 51 ceppi di *Staphylococcus pseudintermedius* isolati da casi clinici di piodermite canina. Tutti gli isolati sono stata testati per la suscettibilità ad un panel di 14 antibiotici con il metodo della diffusione su piastra su Muller Hinton Agar. La produzione di biofilm è stata valutata con il test su piastre microtiter (MtP) e, per alcuni ceppi, con la microscopia elettronica (TEM). Tutti gli isolati di *S. pseudintermedius* sono risultati produttori di biofilm con il metodo MtP; in particolare 17 ceppi sono stati classificati come deboli produttori di biofilm, 26 ceppi come moderatamente produttori di biofilm e 8 come forti produttori di biofilm. Tutti i ceppi hanno mostrato un fenotipo multiresistente; 45 hanno presentato resistenza ad almeno 5 antibiotici contemporaneamente, 6 isolati si sono rivelati resistenti a meno di 6 antibiotici e 4 sono risultati resistenti a tutti i 15 antibiotici testati. Inoltre, i dati ottenuti con la microscopia elettronica sono risultati sovrapponibili a quelli ottenuti con il test su piastre microtiter. L'antibiotico resistenza e la capacità di produrre biofilm potrebbero spiegare il fallimento terapeutico e, conseguentemente, la persistenza dell'infezione sostenuta da *S. pseudintermedius*.

Introduction

Staphylococcus pseudintermedius is a commensal bacterium, which lives in many different animal species and can be an opportunistic pathogen. Indeed, it is mainly responsible for skin infections, such as pyoderma, in dogs and cats (Eckholm *et al.* 2013, Bardiau *et al.* 2013). A dramatic increase in resistance to antimicrobial agents has been observed, particularly with the emergence and the wide-spread dissemination of meticillin-resistant *S. pseudintermedius* (MRSP) in dogs (Song *et al.* 2013). At the same time, in the recent years, the biofilm-forming ability of staphylococci has been accepted as an important virulence trait especially in species as *Staphylococcus epidermidis* and *Staphylococcus aureus* (Smith *et al.* 2008, Rohde *et al.* 2010). Biofilm consists of layers of cell clusters embedded in a matrix of extracellular polysaccharide and adheres to biological or inert surfaces (Vogel *et al.* 2000, Vasudevan *et al.* 2003, Chokr *et al.* 2007, Oliveira *et al.* 2006). The biofilm forming ability of *S. pseudintermedius* has been recently reported, although not fully characterized (Osland *et al.* 2012, Singh *et al.* 2013, Bardiau *et al.* 2013). A recent study showed that the majority of *S. pseudintermedius* isolates from dogs were able to produce biofilm, with 96% being classified as either strong or moderate biofilm producers (Singh *et al.* 2013). Strains belonging to type ST-71, which is the most frequent sequence type in Europe, have been reported to have a significantly greater ability to produce biofilm (Osland *et al.* 2012, Pompilio *et al.* 2015).

Some studies have previously highlighted that bacteria growing within biofilms generally exhibit a greater resistance to antibiotics (Tel *et al.* 2012). Some of the mechanisms linking the antibiotic resistance and biofilm-forming microorganisms, could be the reason making antimicrobials unable to penetrate biofilm, the reason for the slow bacterial growth rate within mature biofilms and, finally, for the induction of a biofilm phenotype characterised by activated multidrug-efflux pumps (König *et al.* 2001, Mah and O'Toole 2001, Stewart and Costerton 2001, Arciola *et al.* 2005). Previous studies have investigated *S. pseudintermedius* biofilm-forming ability (Singh *et al.* 2013) and *S. pseudintermedius* antibiotic resistance (Moodley *et al.* 2014). In the same vain, the aim of the present research was to show a possible correlation between biofilm formation and antibiotic susceptibility in 51 Italian strains of *S. pseudintermedius*, isolated from canine pyoderma.

Materials and methods

Bacterial isolation and identification

A total of 51 canine *S. pseudintermedius* isolates, collected from different dogs with clinical evidence of superficial pyoderma were used in the present study. Pyoderma could be newly diagnosed or recurrent. Clinical abnormalities consistent with superficial pyoderma included papules, pustules, epidermal collarettes, crusts, erythema, and/or hyperpigmentation. Dogs with concurrent illness or current/previous medical therapy, such as antimicrobials, were excluded from the study. Overall, 51 aseptically collected skin samples were sent to the microbiology laboratory, Department of Veterinary Medicine, University of Perugia (Italy) for further analyses.

Clinical samples were cultivated on 5% defibrinated sheep blood agar (Blood Agar Base, Oxoid, Milan, Italy) and Mannitol salt agar (Oxoid, Milan, Italy) and incubated aerobically at 37 °C for 24 hours. Suspect colonies were identified using standard techniques: colony morphology, Gram stain, catalase and coagulase test, and API-Staph System (BioMérieux, Florence, Italy). Methicillin resistant strains were detected by subculture on oxacillin resistant screening agar (ORSA) medium and incubated at 37 °C for 24 hours. Furthermore, all the strains were tested for the presence of methicillin resistance encoding gene *mecA* by polymerase chain reaction (PCR), as previously reported (Zubeir *et al.* 2007). To confirm phenotypic and biochemical results, *S. pseudintermedius* strains were identified using PCR restriction fragment length polymorphism (RFLP) assay based on the *MboI* digestion pattern of a PCR-amplified internal fragment of the *pta* gene, as previously described (Bannoehr *et al.* 2009). All the strains were stored at –70 °C in glycerol storage broth pending further analyses and were aerobically sub-cultured on blood agar plates overnight at 37 °C.

Determination of antibiotic resistance profile

Susceptibility to a panel of 14 antimicrobial agents was determined by the disk diffusion method in Mueller-Hinton agar (Oxoid, Milan, Italy) according to the guidelines of the Clinical Laboratory Standards Institute (CLSI 2015). Discs of cephalexin (CL; 30 µg), ceftriaxone (CRO; 30 µg), penicillin (P; 10 IU), ampicillin (AM; 10 µg), doxycycline (D; 30 µg), amoxicillin-clavulanic acid (AMC; 30 µg), gentamicin (GM; 10 µg), amikacin (AK; 30 µg), enrofloxacin (ENR; 5 µg), trimethoprim-sulfamethoxazole (SXT; 25 µg), and clindamycin (DA; 2 µg) were tested. Marbofloxacin (MAR; 5 µg) was tested according to the methods described by Šeol (Šeol 2005); cefadroxil (CFR; 30 µg) and cefovecin (CVN; 30 µg) were tested following the protocols provided by Westermeyer and collegues (Westermeyer *et al.* 2010). The results were recorded as susceptible, intermediate or resistant by measurement of the inhibition zone diameter.

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Biofilm forming ability assay

Microtitre Plate test (MtP)

The ability of the isolates to form biofilm was investigated by a method described by Stepanovic and colleagues (Stepanovic *et al.* 2000) with minor modifications and was determined by the ability to adhere to 96-well polystyrene microtitre plates (Nunc, ThermoFisher Scientific, Milan, Italy) (Stepanovic *et al.* 2000). Briefly, isolates were sub-cultured onto blood agar, after 24 hours at 37 °C, 1-2 isolate colonies were suspended in 5.0 ml of tryptic soy broth (TSB) supplemented with 1% glucose to achieve a turbidity equivalent to a 0.5 McFarland-standard (~10^8 CFU/ml). A 200 µl bacterial suspension was transferred in triplicates into microtiter plate wells, with the negative control containing growth medium only. Following 24 hours incubation at 37 °C, the contents of the wells were discarded and each well was carefully washed 3 times with sterile phosphate buffered saline (PBS, pH 7.2) to remove non-adherent (planktonic) cells, protecting the integrity of formed biofilms. The adhered bacteria (biofilm) were heat fixed for 60 minutes at 60 °C. Adhered cells were dyed with 150 µl of 0.1% (w/v) crystal violet for 15 minutes at 22 °C, and air dried. After the plates were air dried, the dye bound to the adherent cells was resolubilised with 160 µl of 33% (v/v) glacial acetic acid per well. The optical density (OD) of each well of the microtiter-plate was measured at 570 nm. Each strain was run in triplicate and the results were calculated by the difference between the mean OD570 of the triplicates of each strain and the mean OD570 of the triplicates of the negative control (test broth only). Strains with OD values ≤ 0.065 were regarded as non-adherent, strains with OD values between 0.065 and 0.130 were classified into weakly adherent, strains with OD values between 0.130 and 0.260 were classified into moderately adherent, whereas an OD > 0.260 indicated strains strongly adherent.

Transmission electron microscopy (TEM)

Overnight cultures of 3 strong biofilm producing isolates, 3 moderate biofilm producing strains, and 3 weak biofilm producing strains, randomly chosen, were inoculated with TSB + 1% glucose. The transmission electron microscopy was performed on overnight cultures of biofilm cells fixed as described by Fassel and colleagues (Fassel *et al.* 1997). Bacterial suspensions were centrifuged to remove culture medium, extensively washed, and then fixed. After removing the fixative with centrifugation, the pellet was washed and post-fixed in 2% Osmium tetroxide. Finally, the pellet was dehydrated in a graded series of ethanol up to absolute, it was pre-infiltrated in propylene oxide and then embedded in Epon 812. Ultrathin sections (90 nm) were mounted on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined by means of a Philips EM 208 camera (Centre for Electron Microscopy (CUME), University of Perugia, Perugia, Italy).

Periodic acid thiocarbohydrazide silver proteinate (PATCH)

According to a previous study (Thiéry 1967), PATCH technique was used. This stain technique is analogue to periodic acid-Schiff (PAS) reaction of light microscopy. Bacteria were prepared by fixation in 2.5% glutaraldehyde in Cuprolinic Blue (CB) and post-fixation in Osmium tetroxide. Post-fixation was carried out for 1/2 hour at room temperature to avoid that excessive contrast could cover specific staining of glycoconjugates. The samples were then dehydrated in ethanol and embedded in Epon. Ultrathin sections were mounted on nickel grids and stained in 1% periodic acid for 20 to 30 minutes. After several washes in distilled water, the grids were dipped in 0.2% thiocarbohydrazide in 20% acetic acid and stained at room temperature for 60 minutes in the dark. The sections were washed in graded acetic acid series to water, and then stained for 30 minutes in 1% silver proteinate in double distilled water. After rinsing, the grids were air-dried in the dark.

Statistical analysis

Data derived from laboratory examinations were stored in Microsoft Excel. The isolates of the present study were classified into multidrug-resistant (MDR+) and not multidrug-resistant (MDR-) according to previous studies (Magiorakos *et al.* 2012).

The univariate analysis was initially performed to find a possible statistical association among the following variables: resistance to a single tested antimicrobial agent (i.e., penicillin, etc.), PCR positivity to *mecA* gene, different degree of biofilm (weak, medium, and strong), and MDR+. Chi-squared test, Fisher exact test or Kruskal Wallis, Spearman correlation were calculated. A 2-sided P value <0.05 was considered statistically significant in the univariate analysis.

Factors with P value less than 0.2 in univariate analysis were further used as explanatory variables and investigated in the logistic regression, using the variable multidrug-resistant state (MDR+) as outcome. Possible interactions between explanatory variables were also evaluated. The explanatory variables were manually entered into the analysis. Odds ratios (OR) and corresponding 95% confidence intervals (95% CI) were obtained by

means of logistic regression analysis. The statistical software StatsDirect, version 2.7.9., was used for statistical analysis.

Results

The antibiotic resistance of all 51 *S. pseudintermedius* isolates is summarised in Figure 1. Antibiotic resistance profiling demonstrated that 45 of the 51 *S. pseudintermedius* isolates had a MDR phenotype, exhibiting simultaneous resistance to penicillin (90%), ampicillin (86%), clindamycin (82%), trimethoprim-sulfamethoxazole (80%), enrofloxacin and marbofloxacin (76%). Among these 45 isolates, 4 were resistant to all the 14 tested antibiotics. Six isolates showed non-MDR phenotype. High resistance (59%) was observed toward oxacillin in ORSA medium. In addition, all oxacillin-resistant isolates were positive for *mecA*, indicating that they were *mecA*-mediated MRSP isolates.

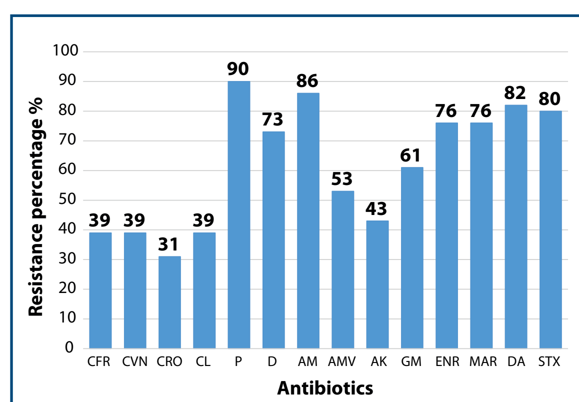

Figure 1. *Antibiotic resistence among* Staphylococcus pseudintermedius *isolates*. CFR = cefadroxil; CVN = cefovecin; CRO = ceftriaxone; CL = cephalexin; P = penicillin; D = doxycycline; AM = ampicillin; AMV = amoxicillin-clavulanic acid; AK = amikacin; GM = gentamicin; ENR = enrofloxacin; MAR = marbofloxacin; DA = clindamycin; SXT = trimethoprim-sulfamethoxazole.

All *S. pseudintermedius* isolates showed biofilm production by MtP method. In particular 17 of the 51 isolates (33%) were classified as weakly, 26 as moderately adherent (51%), and 8 as strongly adherent (16%) (Table I).

At TEM examination, bacteria showed a typical spherical shape, which was more elongated and indented bilaterally in the dividing stage. The cytoplasm was finely granular and moderately electron dense when compared to the wall. Biofilm appeared as a well contrasted, variably (weakly, moderate, and strong) extending fibrillar tree in all samples. The branched fiber-like structures were surrounded by a moderately electron dense amorphous material. The biofilm network seemed to sprout from the bacterial surface, partly adhering to the wall and bridging contiguous bacteria and partly detaching. The PATCH staining revealed the polysaccharidic nature of biofilm and appeared as an array of very small linearly arranged and highly electron-dense spots lying along the fibrillar network (Figure 2).

Moreover, no difference in biofilm formation between MRSP and MSSP (P value > 0.05) was noted. The degree of biofilm was not significantly associated to resistance to each antimicrobial agent or at MDR+

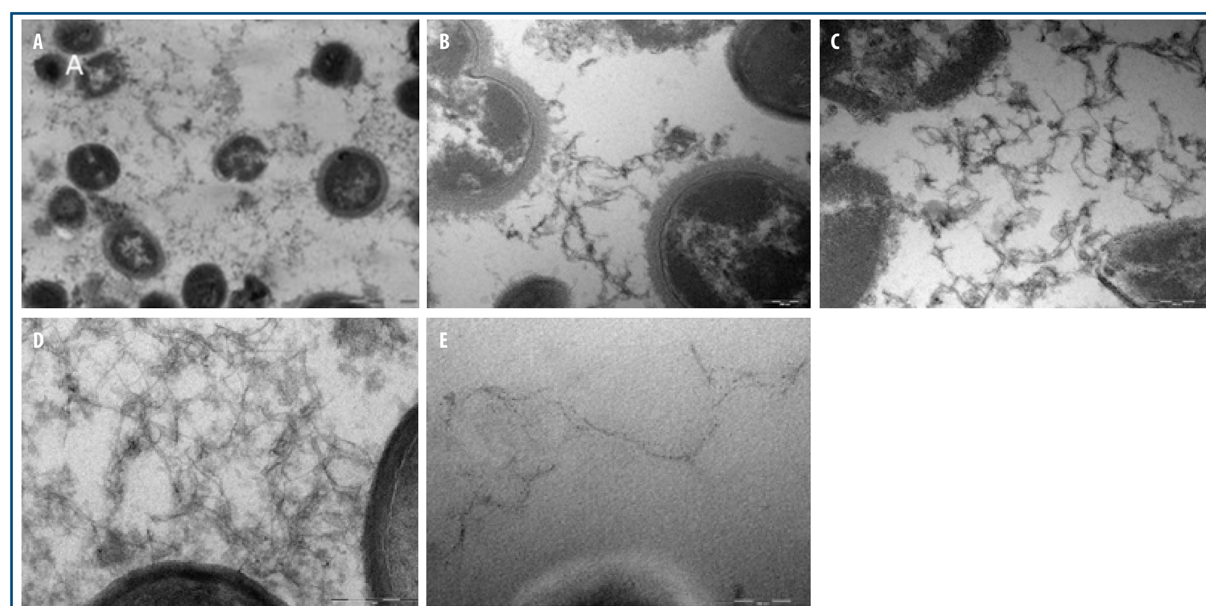

Figure 2. *Transmission electron microscopy of* Staphylococcus pseudintermedius *isolates: (**A**) spatial distribution of extracellular polymeric substances in biofilm; low magnification (**B**) weakly biofilm producer, showing loosely adherent extracellular material, (**C**) moderate biofilm producer and (**D**) strong biofilm producer; (**E**) PATCH staining.*

Table I. *Summary of* Staphylococcus pseudintermedius *isolate profile.*

N.	Antibiotic resistance															Biofilm
	CFR	**CVN**	**CRO**	**CL**	**P**	**D**	**AM**	**AMC**	**AK**	**GM**	**ENR**	**MAR**	**DA**	**SXT**	**OXA**	**MtP test**
1	R	S	S	S	R	R	R	S	R	S	R	R	R	R	S	strong
2	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	moderate
3	S	S	S	S	R	R	S	R	S	S	R	R	R	R	S	moderate
4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	moderate
5	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	moderate
6	S	S	S	S	R	R	R	S	S	S	R	R	R	R	S	moderate
7	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	moderate
8	S	R	S	R	R	R	R	S	S	S	R	R	R	R	S	strong
9	R	R	R	R	R	R	R	R	S	R	R	R	R	R	S	strong
10	R	R	R	R	R	S	R	R	S	R	R	R	R	R	R	moderate
11	R	R	R	R	R	S	R	R	S	R	R	R	R	R	R	strong
12	S	S	S	S	R	R	R	R	S	S	R	R	R	R	S	weak
13	S	S	S	S	R	R	R	S	R	R	R	R	R	R	R	moderate
14	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	moderate
15	S	S	S	S	R	R	R	R	S	S	R	R	R	R	S	weak
16	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	weak
17	S	S	S	S	R	R	R	R	S	S	S	R	S	R	S	moderate
18	R	R	R	R	R	S	R	R	S	S	R	R	R	R	S	moderate
19	S	S	S	S	R	R	R	S	R	R	R	R	R	S	R	moderate
20	S	S	S	S	R	R	R	S	S	S	R	R	R	S	S	weak
21	S	S	S	S	R	R	R	S	R	R	R	R	R	S	R	strong
22	R	R	S	S	R	R	R	R	R	R	R	R	R	R	S	moderate
23	S	S	S	S	R	S	R	S	S	S	R	R	R	R	S	weak
24	S	S	S	S	R	R	R	R	R	R	R	R	R	R	S	moderate
25	S	S	S	S	S	S	S	S	R	R	R	R	R	R	R	moderate
26	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	weak
27	S	S	S	R	R	R	R	R	R	R	R	R	R	R	S	moderate
28	S	S	S	S	R	S	R	S	S	S	S	S	R	R	S	moderate
29	S	S	S	S	R	S	R	S	R	R	R	R	S	R	R	weak
30	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	weak
31	R	R	S	R	R	R	R	S	S	S	S	S	R	R	R	weak
32	R	R	S	R	R	R	R	S	S	S	R	R	R	R	R	moderate
33	S	S	S	S	S	R	S	S	R	R	R	R	R	R	R	moderate
34	S	S	S	S	R	S	R	S	R	R	S	S	S	S	R	moderate
35	S	S	S	S	R	R	S	S	R	R	R	R	S	R	S	moderate
36	S	S	S	S	R	R	R	S	R	R	R	R	R	R	R	weak
37	S	S	S	S	R	S	R	S	S	S	S	S	S	S	S	weak
38	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	weak
39	R	R	R	R	R	R	R	R	S	S	S	S	S	R	R	moderate
40	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	moderate
41	S	S	S	S	S	S	S	S	S	S	S	S	R	S	R	weak
42	S	S	S	S	R	R	R	S	R	R	R	R	R	R	S	strong
43	S	S	S	S	R	S	R	S	R	R	R	S	R	S	R	weak
44	S	S	S	S	S	S	S	S	R	R	S	S	S	S	S	weak
45	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S	moderate
46	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	moderate
47	S	S	S	S	R	S	R	R	S	S	S	S	R	S	S	weak
48	S	S	S	S	R	R	R	R	R	R	R	R	R	R	R	weak
49	S	S	S	S	R	R	R	R	S	S	R	S	S	S	R	moderate
50	S	S	S	S	R	R	R	R	S	S	S	S	S	R	R	strong
51	S	S	S	S	R	R	R	R	R	R	S	R	R	R	R	strong

R = resistant; S = sensible; CFR = cefadroxil; CVN = cefovecin; CRO = ceftriaxone; CL = cephalexin; P = penicillin; D = doxycycline; AM = ampicillin; AMV = amoxicillin-clavulanic acid; AK = amikacin; GM = gentamicin; ENR = enrofloxacin; MAR = marbofloxacin; DA = clindamycin; SXT = trimethoprim-sulfamethoxazole.

(*P* value > 0.05), while the presence of *mecA* gene was associated to MDR+, as well as to resistance to all tested cephalosporins and gentamycin. All MDR+ isolates were PCR positive to *mecA* gene. The isolates that were PCR negative to *mecA* gene were quite equally distributed between MDR+ and MDR-.

At the same time, MDR+ was statistically associated with resistance to all tested cephalosporins and gentamicin. However, only resistance to doxycycline and gentamycin showed to be significant at the multivariable analysis, with an odds ratio of 14.5 (Confidence Interval, CI 95%: 2.2-95.6) for doxycycline and 12.5 (CI 95%: 1.7-91.6) for gentamycin. No interaction among resistance to different antimicrobials was observed. The resistance to the other antimicrobial agents and PCR positivity to *mecA* gene were not detected from multivariable models, accordingly they were not predictors of MDR+ in the multivariable analysis.

Discussion

Antibiotic resistance is one of the important problems encountered in treatment and control of *S. pseudintermedius* infection. Pyoderma caused by resistant bacteria is difficult to cure and has severe consequences. In this study, we firstly investigated the differences in the prevalence of antibiotic resistance by agar diffusion test. Results showed that *S. pseudintermedius* isolates had high antibiotic resistance.

Furthermore, MDR was defined as acquired non-susceptibility to at least 1 agent in 3 or more antimicrobial categories (Magiorakos *et al.* 2012). Forty-five out 51 *S. pseudintermedius* isolates had MDR phenotype, whereas 6 isolates had not-MDR phenotype. Moreover MRSP rate was higher (59%) than the one reported in other studies, (Sasaki *et al.* 2007, Hanselman *et al.* 2009, Weese and van Duijkeren 2010, Onuma *et al.* 2012), including our own (Casagrande Proietti *et al.* 2012).

Biofilm formation has increasingly been accepted as an important virulence factor (Vancraeynest *et al.* 2004) and it is important for bacterial persistence and survival in the environment. In this study we investigated the *S. pseudintermedius* biofilm-forming ability by MtP and by TEM. The MtP method revealed that all *S. pseudintermedius* isolates were biofilm producers. Of these, 17 (33%) were weak, 26 (51%) moderate, and 8 (16%) strong biofilm phenotypes. These data were in contrast with those provided by Singh and colleagues (Singh *et al.* 2013), whose study observed that, by using MtP, the majority of isolates (61%) were strong biofilm producers, while few isolates (3%) had weak biofilm phenotypes (34% moderate and 2% non-producers). At the same time, we did not detect any difference in biofilm formation between MRSP and MSSP, confirming the results described in the same study (Singh *et al.* 2013).

In our study 3 weak, 3 moderate, and 3 strong biofilm producer isolates were randomly chosen from collection and were used for biofilm structure evaluation by TEM, which confirmed the expected phenotypes. Even though the MtP method is considered a reliable and sensitive quantitative technique for biofilm screening, the electron microscopy is one of the golden standards to show biofilm formation. However, electron microscopy is work-intensive and requires an expensive equipment and expertise, so it is not diffusely used for screening or routine diagnosis, although it could be useful to understand biofilm structure and complexity.

Our results showed that all *S. pseudintermedius* isolates were biofilm producers and the majority of the isolates (45/51) had MDR phenotype, although no statistical association between the degree (week, moderate, strong) of biofilm and antibiotic resistance was observed. Actually, the degree of biofilm was not significantly associated to any variable analysed in the present study. The presence of *mecA* gene was associated with resistance to all tested cephalosporins, gentamycin, and MDR+ in univariate analysis.

However, multivariable analysis showed that only the resistance to doxycycline and gentamycin were predictors of MDR+ isolates. Other factors should be investigated to explain the antimicrobial resistance of the isolates.

As supported by previous studies, the low susceptibility to antibiotics appears to be attributed to insufficient penetration of antibiotics into biofilm, to the reduced growth rate of bacteria embedded in biofilm, and to the wide variety of altered metabolic states within the biofilms that are necessary for cell survival in hostile environments (Costerton *et al.* 1999). Although in the present study the degree of biofilm was not significantly associated to resistance to each antimicrobial agent or at MDR+, it is important to underline that all the *S. pseudintermedius* strains were biofilm-producers and that 45 out of 51 strains had a MDR phenotype. Within a biofilm, bacteria are able to evade the host immune response and antimicrobials effects through physical and chemical protection of the biofilm matrix (Singh *et al.* 2013).

In conclusion, the high antimicrobial resistance rate in combination with biofilm production could explain the difficulty of treating *S. pseudintermedius* canine infections, chemotherapeutic failure and, consequently, the rapid emergence of this bacterium in veterinary hospitals worldwide. All these elements lead to consider biofilm formation as a relevant marker of microbial virulence as

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well as an indicator of potentially dangerous strains, generally less sensitive to antibiotic treatment. Furthermore, there is evidence of MRSP transmission to humans, suggesting a possible zoonotic potential (Riegel *et al.* 2011, Pompilio *et al.* 2015) so further studies are certainly required to expand our knowledge about *S. pseudintermedius* infection control and virulence.

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