Occurrence and antimicrobial susceptibility patterns of canine Staphylococcus pseudintermedius *strains isolated from two different Italian university veterinary hospitals*

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Summary
Staphylococcus pseudintermedius represents one of the most frequently bacteria isolated on dog's skin and it was recently recognized as a zoonotic pathogen responsible for severe diseases also in humans. This study aimed to define the occurrence of canine methicillin-resistant and methicillin-susceptible *S. pseudintermedius* (MRSP and MSSP) strains and to compare their antimicrobial profiles. The study was carried out at veterinary microbiology laboratories of two different Italian veterinary teaching hospitals, Milan and Naples, from 2015 to 2017. The statistical comparison of the results revealed significant differences in MRSP occurrence (p-value = 0.0435) and MRSP and MSSP antibiotic resistance profiles. In Milan, MRSP strains displayed significantly higher antibiotic resistance percentages ($p < 0.001$) for some antibiotics, such as ceftriaxone and tobramycin, compared to those of Naples. Conversely, MSSP strains of Naples presented significantly higher rates ($p < 0.001$) of resistance to amoxicillin/clavulanic acid, kanamycin, erythromycin, and tetracycline than to Milan isolates. In conclusion, the results highlighted relevant variances among region-specific antibiotic resistance profiles, probably due to different antimicrobial selection pressures. Therefore, this study stands out the need for continuous monitoring of both MRSP and MSSP linked to different geographical areas, also considering their impact and importance on animal and human health.

Introduction

Staphylococcus pseudintermedius (*S. pseudintermedius*) has been described for the first time in 2005 in dogs and pigeons, thanks to the 16S rRNA gene sequence analyses (Devriese *et al.* 2005).

S. pseudintermedius can be isolated in healthy dogs from forehead, nares, oral mucosa, pharynx, groin, and anus (Garbacz *et al.* 2013). However, in particular conditions, such as injuries and sickness, it can act as an opportunistic pathogen for dogs and cats and, mainly in dogs, *S. pseudintermedius* is associated with skin and ear infections (De Martino *et al.* 2016, Fitzgerald 2009, Weese and van Duijkeren 2010, van Duijkeren *et al.* 2011).

In the past, *S. pseudintermedius* isolates were generally susceptible to β-lactams, whose principal antimicrobial agent is penicillin. Therefore, methicillin-susceptible *S. pseudintermedius* (MSSP) strains originally circulated in canine population. However, already in 2006, methicillin-resistant *S. pseudintermedius* (MRSP) strains were isolated in Europe, becoming a relevant problem in veterinary medicine.

Over the years, MRSP have been reported with an increasing frequency (Loeffler *et al.* 2007, van Duijkeren *et al.* 2011, Kasai *et al.* 2016) and interestingly MRSP strains have often been showing multidrug resistance profiles worldwide, including resistance to several classes of antimicrobial drugs

(Perreten *et al.* 2010). This scenario limits the treatment options and represents a relevant threat to small animal therapy in veterinary medicine, challenging infection control measures (van Duijkeren *et al.* 2011, Bannoehr and Guardabassi 2012, Bond and Loeffler 2012). There are several reports on isolates resistant almost to all antimicrobials authorized in veterinary medicine (Wettstein *et al.* 2008, Perreten *et al.* 2010), inducing clinicians to use antimicrobials authorized only for human medicine (Weese and van Duijkeren 2010).

The *S. pseudintermedius* virulence potential and its zoonotic transmission should not be underestimated, even though it is not often reported. *S. pseudintermedius*, particularly MRSP, has also been isolated from humans, especially in pet owners (Van Hoovels *et al.* 2006, Stegmann *et al.* 2010, Somayaji *et al.* 2016, Robb *et al.* 2017, Lozano *et al.* 2017). As a member of the *Staphylococcus* genus, *S. pseudintermedius* has an extensive panel of virulence factors (van Duijkeren *et al.* 2011). The majority of these are firstly involved in the colonization of host tissues and the dissemination in the colonized district. Specifically, exfoliative toxins (*siet*, *expA*, and *expB*) are virulence factors involved in canine pyoderma (Futagawa-Saito *et al.* 2009, Iyori *et al.* 2010, Yoon *et al.* 2010).

This study aimed to collect canine *S. pseudintermedius* strains, in order to define the occurrence of MRSP and MSSP isolated from dogs suffering from skin disorders in two different Italian veterinary teaching hospitals; moreover, the antibiotic resistance profiles among both MRSP and MSSP isolates were compared.

Materials and methods

Sample collection

Between 2015 and 2017, *S. pseudintermedius* strains were isolated from routine bacteriological examinations of canine samples. More in detail, clinical samples, represented by auricular and cutaneous swabs, were collected from dogs suffering from skin disorders, visiting one of the two university veterinary hospitals, located in Milan and Naples.

Isolation and phenotypic identification of *S. pseudintermedius*

Upon arrival at the laboratories, canine auricular and skin samples were cultured and streaked on Columbia CNA agar plates (Liofilchem, Italy), then incubated aerobically at 37 °C for 24 h. Suspected staphylococcal colonies were firstly identified by standard, rapid screening techniques: colony morphology, β-haemolysis, cellular morphology (Gram's staining), catalase test, and then sub-cultured on Mannitol Salt Agar plates (MSA, Liofilchem, Italy). Each mannitol salt negative colony was also subjected to staphylocoagulase (tube coagulase) test (Oxoid, Ltd, UK) to confirm their capacity to produce coagulase enzymes. The identification was assessed by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis (Bruker Daltonics, Germany), using fresh colonies. Values from 2.3 to 1.9 indicated the best identification of genus and species of staphylococci (Santos *et al.* 2013, Nisa *et al.* 2019). All the strains were stored in 25% glycerol until use (Carlo Erba, Italy) at - 20 °C. Before use, samples were thawed at room temperature, and 10 µL were plated on Tryptic Soy Agar + 5% sheep blood (Microbiol, Italy) and incubated aerobically at 37 °C for 24 h. Three or four isolated colonies were picked up and used for the analyses.

Genetic identification of *S. pseudintermedius*

For the molecular identification of the selected strains, DNA was extracted from fresh cultures of each *S. pseudintermedius* isolates. The bacterial DNA extraction was carried out by the boiling method (Adwan *et al.* 2014). The quantity and quality of DNA were determined by the spectrophotometric reading of A260/A280 ratio (Eppendorf BioPhotometer 6131). Then, DNA samples were stored at - 20 °C.

Molecular identification, using species-specific *nuc* gene (Sasaki *et al.* 2010) was performed by PCR to confirm the identification of *S. pseudintermedius* strains. DNA of *S. pseudintermedius* ATCC® 49444TM was used as a positive control.

Antimicrobial susceptibility test

The antimicrobial susceptibility test was performed on all *S. pseudintermedius* strains using Kirby-Bauer disk diffusion method according to Clinical Laboratory and Standards Institute (CLSI 2015) guidelines. Strains were classified as susceptible, intermediate and resistant by comparison of the zone of inhibition as indicated by the same guidelines. Table I reported the antibiotics used .

Amplification of antibiotic-resistance genes (ARg)

To study the dissemination of genes coding for antimicrobial resistance, the amplification of the following genes was performed by qualitative PCRs: *mec*A (Chovanovà *et al.* 2015), *mec*C (Stegger *et al.* 2012) and tetracyclines family genes (*tet*K, *tet*L, *tet*M,

Table I. *Tested antibiotics against methicillin-resistant* S. pseudintermedius *(MRSP) and methicillin-susceptible* S. pseudintermedius *(MSSP) in Milan and Naples Labs.*

Group	Antibiotics (µg)	MRSP (% R)* Milan / Naples	MSSP (% R) Milan / Naples
Penicillins (β-lactams)	AMC (20/10 µg)	88.6 / 100	7.4 / 56.3***
Penicillins (β-lactams)	OX (1 µg)	100 / 100	0.0 / 0.0
Cephalosporins (β-lactams)	CRO (30 µg)	91.4 / 47.8***	1.2 / 12.6**
Lincosamides	DA (2 µg)	97.1 / 65.2*	32.2 / 33.0
Fluoroquinolones	ENR (5 µg)	85.7 / 60.9	17.2 / 16.5
Macrolides	E (15 µg)	100 / 91.3	7.4 / 36.8***
Aminoglycosides	CN (10 µg)	48.5 / 52.2	2.4 / 9.7
Aminoglycosides	K (30 µg)	100 / 78.3**	7.4 / 43.6***
Aminoglycosides	TOB (10 µg)	80.0 / 13.0***	6.1 / 3.8
Tetracyclines	TE (30 µg)	71.4 / 87.0	19.7 / 50.5***

AMC = amoxicillin + clavulanic acid; OX = oxacillin; CRO = ceftriaxone; DA = clindamycin; ENR = enrofloxacin; E = erythromycin; CN = gentamicin; K = kanamycin; TOB = tobramycin; TE = tetracycline.
(% R) = Percentage of resistance. χ^2: *$0.05 < p < 0.01$; **$p < 0.01$; ***$p < 0.001$.

and *tet*O) (Ullah *et al.* 2012). Furthermore, other ARg were searched in both laboratories; *aac*A-*aph*D (Strommenger *et al.* 2003) and *bla*Z (Kang *et al.* 2014) in Milan laboratory; while *erm*A, *erm*B, *erm*C (Sutcliffe *et al.* 1996) in Naples. Both positive and negative appropriate controls were used in all PCR experiments.

Statistical analysis

Samples were grouped in MRSP and MSSP, according to the results of oxacillin disk diffusion test and the presence of *mec* gene. The tested antibiotics (N = 10) and the genes of tetracycline resistance, studied both in Milan and Naples, were statistically analyzed with the chi-square test to verify the difference in the distribution of phenotypic and genotypic resistance profiles.

Chi-square test was performed with GraphPad Prism (version 8.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

Results

S. pseudintermedius isolation and identification

Between 2015 and 2017, 242 *S. pseudintermedius* strains were collected from canine skin disorders (pyoderma and otitis externa), particularly 116 strains in Milan and 126 in Naples. All the strains showed β-haemolytic patterns on Colombia CNA agar and inability to ferment mannitol on MSA. Furthermore, they showed positive results at the staphylocoagulase and catalase tests. The bacterial identification results obtained by MALDI-TOF MS were confirmed by genetic identification using the species-specific nuc gene, revealing a clear concordance between molecular and proteomic analyses in both laboratories.

Antimicrobial susceptibility testing

The phenotypical oxacillin resistance, investigated by disk diffusion test, was confirmed by the amplification of *mec*A gene and allowed to distinguish between MRSP and MSSP strains. No detection of *mec*C gene was revealed among all screened strains. The prevalence of MRSP was significantly higher in Milan 30% (35/116) than that reported in Naples 18% (23/126), resulting in statistical significance (p-value = 0.0435).

As shown in Table I, in Milan and Naples, the antimicrobial susceptibility profiles of the MRSP strains showed high resistance to amoxicillin-clavulanate being 88.6% and 100%, respectively. However, the overall prevalence of antimicrobial resistance among the MRSP isolates detected in this study appeared high. In Milan, all the MRSP strains were found to be multidrug resistant strains showing resistance to at least three different antibiotic classes, while in Naples, 91% of MRSP strains with multidrug resistant profiles were observed.

Furthermore, in Milan a significantly higher percentage of MRSP isolates were resistant to ceftriaxone and tobramycin compared to those in Naples ($p < 0.001$). Clindamycin and kanamycin resistances also appeared to be statistically higher in Milan than in Naples ($p < 0.05$ and $p < 0.01$, respectively). This investigation also revealed that MRSP strains showed markedly high resistance to enrofloxacin, erythromycin, tetracycline in both groups of MRSP strains. Similar results were also observed for resistance to gentamicin (48.5% Milan, 52.2% Naples).

In both laboratories, the phenotypic profiles of antibiotic resistance showed lower percentages of resistance to MSSP strains than MRSP ones (Table I). The MSSP strains isolated in Naples harbored the highest prevalence of resistance to amoxicillin/clavulanic acid (56.3%), erythromycin (36.8%), kanamycin (43.6%) and tetracycline (50.5%). Conversely, those isolated in Milan showed statistically significant lower percentages ($p < 0.001$) of resistence to the same antibiotics, 7.4% (amoxicillin/clavulanic acid), 7.4% (erythromycin), 7.4% (kanamycin) and 19.7% (tetracycline). A significant difference in ceftriaxone resistance was also observed between Milan and Naples ($p < 0.01$) with a higher prevalence in the second

hospital. Moreover, in Milan, 7% of MSSP strains were multidrug resistant and 45% of MSSP resulted susceptible to all tested antibiotics; in Naples, 34% of MSSP were multidrug resistant and, among them, 10% resulted susceptible to all tested antibiotics.

The phenotypic tetracycline-resistant MRSP strains of Milan (n = 25) and Naples (n = 20) harbored *tet*K and *tet*M genes, alone or in combination, as well as the MSSP, that were exactly 16 and 52 strains, respectively. Genotypic characterization of tetracycline resistance for the samples from Milan showed a higher rate of *tet*M gene for both MRSP (56%) and MSSP (81.2%). Similar results were also evidenced for the strains of Naples, 52% and 46.1%, respectively (Table II). Only among MSSP strains, the differences in the frequency of *tet*M gene between Milan and Naples resulted in being statistically significant with a *p*-value < 0.05 (Table II). The tetracycline-resistant strains, carrying *tet*K and *tet*M together showed the lowest prevalence among the isolates of both laboratories and no significant differences between Milan and Naples results were observed (Table II).

Besides, in Milan, all the isolates positive to *mec*A gene harbored also *bla*Z gene, and the gentamicin resistance was confirmed with the presence of the gene *aac*A/*aph*D (data not shown); while in Naples, all the phenotypic erythromycin-resistant strains carried *erm*B gene (data not shown).

Table II. *Molecular profiles of tetracycline resistance in canine* S. pseudintermedius *isolates.*

Molecular profiles of tetracycline resistance	MRSP-Milan	MRSP-Naples	MSSP-Milan	MSSP-Naples
*tet*K gene	10/25 (40.0%)	6/20 (26%)	2/16 (12.5%)	17/52 (32.7%)
*tet*M gene	14/25 (56.0%)	12/20 (52%)	13/16 (81.2%)	24/52 (46.1%)*
*tet*K and *tet*M genes	1/25 (4.0%)	2/20 (9%)	1/16 (6.3%)	11/52 (21.2%)

χ^2: * *p*-value < 0.05; MSSP = methicillin-susceptible *S. pseudintermedius*; MRSP = methicillin-resistant *S. pseudintermedius*.

Discussion

Bacterial otitis externa and pyoderma are the most common canine skin diseases, and *S. pseudintermedius* is the staphylococcal species most frequently isolated from dogs suffering from these infections. This bacterium, an opportunistic canine skin pathogen, is the major coagulase positive (CPS) agent, that inhabits healthy dogs (Gómez-Sanz *et al.* 2013). In particular conditions, such as dog injures or sickness, this species can take advantage of the weakened host immune defenses and cause infection and illness.

Antibiotic resistance is the most puzzling question in recent years, and the spread of multidrug resistant staphylococci of animal origin, principally MRSP strains, has increased its public health relevance (Deurenberg *et al.* 2007, Corrente *et al.* 2013). In this context, the emergence in dogs of MRSP, often associated with even broader drug resistance, has become a great veterinary challenge. In this study, of the 242 isolated *S. pseudintermedius* strains, 30% (33/116 in Milan) and 18% (23/126 in Naples), were MRSP presenting almost all worrying multidrug resistant profiles. Besides β-lactam antibiotics, the multidrug resistance profile of MRSP strains showed relevant resistance rates to all classes of antibiotics approved in veterinary medicine and used for systemic treatment in dogs (tetracycline, aminoglycosides, macrolides, sulfamethoxazole-trimethoprim, lincosamides), confirming the multidrug resistance profiles reported for MRSP worldwide (Osland *et al.* 2012, Haenni *et al.* 2014, Moodley *et al.* 2014, Stefanetti *et al.* 2017).

It is known that MRSP originates from an animal reservoir; consequently, pet animals might act as potential reservoirs for the emergence of novel methicillin-resistant clones in humans. Furthermore, in recent years, it has been reported an increase of the zoonotic transmission of MRSP, probably due to more appropriate identification of this strain (Somayaji *et al.* 2016, Robb *et al.* 2017, Lozano *et al.* 2017).

The veterinary environments seem to play an essential role in the dissemination of MRSP between pet animals and humans, particularly people who have constant contact with pets (veterinary personnel and pet owners) (Paul *et al.* 2011, van Duijkeren *et al.* 2011).

In this study, interesting antibiotic resistance profiles were also shown by MSSP strains, although their resistance profiles were lower compared to those reported by MRSP. The MSSP strains from Naples displayed a significantly higher resistance rate compared to the strains from Milan and also in comparison to the available literature (Ganiere *et al.* 2005, Norström *et al.* 2009). However, the reported increasing antibiotic resistance percentage of MSSP strains from Naples is also confirmed by more recent studies in other European countries (Haenni *et al.* 2014, Moodley *et al.* 2014). The MSSP strains showed high resistance rates to antibiotics commonly used to treat canine infections, such as the penicillinase-labile penicillins, tetracycline, aminoglycosides, and macrolides. It is worth noting that in Naples, 34% of MSSP were multidrug resistant strains, while only 10% of MSSP isolates were susceptible to all tested antibiotics.

Furthermore, in accordance with the literature, this study shows that *tet*K and *tet*M genes were

the most prevalent tetracycline resistance determinants in both MRSP and MSSP (Schmitz *et al.* 2001, Youn *et al.* 2014). Moreover, for both MRSP and MSSP strains isolated in the laboratory of Naples, all the erythromycin-resistant strains harbored only *erm*B gene, that usually appears to be responsible for erythromycin resistance in canine *S. pseudintermedius* strains (Boerlin *et al.* 2001, Lüthje and Schwarz, 2007, Youn *et al.* 2014).

MRSP strains are known to be more resistant than MSSP strains, and their high prevalence of multidrug resistance is probably related to the dissemination of dominant clones, owning specific antibiotic resistance profiles and virulence genes (for instance, MRSP belonging to the clonal lineage ST71) (Moodley *et al.* 2014). However, the observed results on MSSP antibiotic resistance highlight that the spread of multidrug resistant MSSP should be monitored, and their pathogenic role deserves further studies.

In conclusion, the increasing circulation of both MRSP and MSSP strains with worrying antibiotic resistance profiles and the lack of effective new antibiotics highlight the limited therapeutic possibilities and the need for new treatment approaches to prevent and control staphylococcal infections. Another fundamental aspect of this study is the necessity to monitor the antibiotic resistance profiles in different territorial areas. Understanding the differences in antimicrobial resistance profiles and specific resistance gene carriage by *S. pseudintermedius* strains isolated in different geographic regions may improve antimicrobial drug selection overall for clinical therapy and, consequently, provide insights into how resistance develops in both MRSP and MSSP.

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