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Original article

Characterization of *Staphylococcus pseudintermedius* isolated from diseased dogs in Lithuania

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Abstract

The aim of this study was to characterize *Staphylococcus pseudintermedius* for its antimicrobial resistance and virulence factors with a special focus on methicillin-resistant (MRSP) strains isolated from sick dogs in Lithuania. Clinically sick adult dogs suffering from infections (n=214) and bitches with reproductive disorders (n=36) from kennels were selected for the study. Samples (n=192) from the 250 tested (76.8%) dogs were positive for *Staphylococcus* spp. Molecular profiling using the species-specific *nuc* gene identified 51 isolates as *S. pseudintermedius* (26.6% from a total number of isolated staphylococci) of which 15 isolates were identified as MRSP. Ten MRSP isolates were isolated from bitches with reproductive disorders from two large breeding kennels. Data on susceptibility of *S. pseudintermedius* to different antimicrobials revealed that all isolates were susceptible to vancomycin, daptomycin and linezolid. Two isolates (3.9%) were resistant to rifampicin. A high resistance was seen towards penicillin G (94.1%), tetracycline (64.7%) and macrolides (68.7%). Resistance to fluoroquinolones ranged from 25.5% (gatifloxacin) to 31.4% (ciprofloxacin). The most prevalent genes encoding resistance included *bla*Z, *aac(6')-Ie-aph(2'')-Ia*, *mec*A, and *tet*(M). The Luk-I gene encoding a leukotoxin was detected in 29% of the isolates, whereas the *siet* gene encoding exfoliative toxin was detected in 69% of the *S. pseudintermedius* isolates. This report of MRSP in companion animals represents a major challenge for veterinarians in terms of antibiotic therapy and is a concern for both animal and public health.

Key words: MRSP, companion animals, resistance, antimicrobials, toxins

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Introduction

Staphylococcus pseudintermedius is an important opportunistic pathogen of companion animals, especially dogs (van Duijkeren et al. 2011). Canine infections caused by *S. pseudintermedius* are mostly skin infections, endometritis, cystitis and other less frequent infections (Cox et al. 1984, Morris et al. 2006). *S. pseudintermedius* has various virulence factors, including some that are closely related to the virulence factors of *S. aureus* (Futagawa-Saito et al. 2004b, Fitzgerald 2009). It produces enzymes such as coagulase, protease, thermonuclease and toxins, including haemolysins and exfoliative toxins (Fitzgerald 2009, Ben Zakour 2011). *S. pseudintermedius* also produces a leucotoxin known as Luk-I, which is very similar to Panton-Valentine leucocidin (PVL) from *S. aureus* (Futagawa-Saito et al. 2009, Futagawa Saito et al. 2004a).

Methicillin-resistant *S. pseudintermedius* (MRSP) has posed an increasing therapeutic challenge because of its limited treatment options. Colonization and infection caused by MRSP has been described in dogs, cats, horses, birds and humans. This fact demonstrates the zoonotical potential of *S. pseudintermedius*. It is also known that dogs can carry the same or similar MRSP strains for months without active infection. Several reports on isolates not susceptible to any antimicrobials authorized for use in veterinary medicine have been published causing veterinarians to consider using antimicrobials authorized for human medicine only. Good veterinary practice requires the use of antimicrobial treatment after correct diagnosis and susceptibility testing. However, reliable commercial identification systems for fast and correct identification of *S. pseudintermedius* are not currently available. In many cases, isolates will be erroneously identified as *S. intermedius* or *S. aureus*.

MRSP isolates are characterized by the presence of the *mecA* gene, which is located on staphylococcal cassette chromosome *mec* (SCC*mec*) elements and confers resistance to all f-lactam antibiotics. In addition to *mecA*, MRSP also may contain a wide range of antibiotic resistance genes. In addition to β-lactam resistance, resistance to 11 other antimicrobials was observed in a study of 103 epidemiologically unrelated MRSP isolates from dogs from Canada, the USA, Denmark, Germany, France, Italy, Sweden, Switzerland and the Netherlands. The resistance of *S. pseudintermedius* depends on geographical distribution as well as on other factors – thus, it is important to obtain data from different countries to better understand the epidemiological spread of resistance.

The aim of this study was to characterize *Staphylococcus pseudintermedius* in sick Lithuanian dogs for antimicrobial resistance and virulence factors with a special focus on methicillin-resistant (MRSP) strains.

Materials and Methods

The investigations were carried out at the Lithuanian University of Health Sciences, Institute of Microbiology and Virology.

Sample collection

Two hundred and fifty dogs were randomly selected from small animal clinics located throughout the country as well as in breeding kennels of pure-breed dogs. Clinically sick adult dogs suffering from skin infections (n=155), otitis (n=48), respiratory tract infections (n=11) and bitches with reproductive disorders (metritis, temporal infertility, premature birth) (n=36) were selected for the study. The age ranged between 2 and 8 years. Anamnesis data showed that 190 dogs were untreated with antimicrobials at least 6 months before sampling; 45 sick dogs were treated over different intervals during the last 0-30 days before sampling. Fifteen bitches in kennels were prophylactically treated with fluoroquinolones and/or cephalosporins during the previous 3 months. Sterile cotton swabs with transport media (TRANSWAB, Polysciences Inc.) or sterile syringes were used for collection of clinical samples. These samples were delivered to the laboratory on the same day. Only one sample from the affected organ was taken from each dog.

Bacteriological testing and DNA extraction

Samples were inoculated onto Mannitol-Salt Agar (Liofilchem, Italy) and Mannitol-Salt Agar supplemented with oxacillin (Sigma-Aldrich). Suspected colonies of *Staphylococcus* spp. were tested for presumptive genus identification according to the production of haemolysis, catalase, gram-staining, susceptibility to furazolidone, morphology and growing characteristics followed by a latex agglutination test („Staph Latex Kit", Microgen, UK). Presumptive *S. pseudintermedius* isolates were identified up to species level using the RapID STAPH PLUS (Thermo Scientific) identification system and ERIC® manufacturer's software.

DNA material for molecular testing was obtained after bacterial lysis according to the extraction protocol prepared by the Community Reference Labora-

Table 1. Oligonucleotide primers used in this study.

Primer name	Sequence (5' – 3')	Size, bp and T (°C)	Target gene	Source
nuc1 nuc2	TRGGCAGTAGGATTCGTTAA CTTTTGTGCTYCMTTTTGG	926 (58)	*nuc*	Sasaki et al. 2010
siet1 siet2	ATGGAAAATTTAGCGGCATCTGG CCATTACTTTTCGCTTGTTGTGC	359 (56)	exfoliative toxin	Lautz et al. 2006
lukS1 lukS2	TGTAAGCAGCAGAAAATGGGG GCCCGATAGGACTTCTTACAA	503 (57)	*luk*S	Futagawa-Saito et al. 2004-1
lukF1 lukF2	CCTGTCTATGCCGCTAATCAA AGGTCATGGAAGCTATCTCGA	572 (57)	*luk*F	Futagawa-Saito et al. 2004-1
mecA1 mecA2	GGGATCATAGCGTCATTATTC AACGATTGTGACACGATAGCC	527 (61)	*mec*A	Poulsen et al. 2003
mecC1 mecC2	GCTCCTAATGCTAATGCA TAAGCAATAATGACTACC	204 (50)	*mec*C	Cuny et al. 2011
blaZ1 blaZ2	CAGTTCACATGCCAAAGAG TACACTCTTGGCGGTTTC	772 (50)	*bla*Z	Schnellmann et al. 2006
tetM1 tetM2	GTTAAATAGTGTTCTTGGAG CTAAGATATGGCTCTAACAA	656 (45)	*tet*M	Aarestrup et al. 2000
tetK1 tetK2	TTAGGTGAAGGGTTAGGTCC GCAAACTCATTCCAGAAGCA	718 (55)	*tet*K	Aarestrup et al. 2000
aac6-aph2F aac6-aph2R	CAGAGCCTTGGGAAGATGAAG CCTCGTGTAATTCATGTTCTGGC	348 (61)	*aac(6')-Ie-aph(2'')-Ia*	Perreten et al. 2005
aph3-IIF aph3-IIR	CCGCTGCGTAAAAGATAC GTCATACCACTTGTCCGC	609 (57)	*aph(3')-IIIa*	Perreten et al. 2005
dfrK1 dfrK2	GCTGCGATGGATAAGAACAG GGACGATTTCACAACCATTAAAGC	214 (50)	dfrK	Kadlec and Schwarz 2010
ermA1 ermA2	AAGCGGTAAAACCCCTCTGAG TCAAAGCCTGTCGGAATTGG	442 (53)	*erm*A	Jensen et al. 2002
ermC1 ermC2	ATCTTTGAAATCGGCTCAGG CAAACCCGTATTCCACGATT	295 (48)	*erm*C	Jensen et al. 2002
ermB1 ermB2	GGAACATCTGTGGTATGGCG CATTTAACGACGAAACTGGC	425 (48)	*ermB*	Jensen et al. 2002
msrA1 msrA2	GCTTAACATGGATGTGG GATTGTCCTGTTAATTCCC	1230 (55)	*msr*A	Perreten et al. 2005
16S1 16S2	GTGCCAGCAGCCGCGGTAA AGACCCGGGAACGTATTCAC	886 (61)	*16S* staph	Poulsen et al. 2003

tory for Antimicrobial Resistance with slight modifications. Briefly, bacterial colonies were taken with a bacteriological loop from the surface of Mueller Hinton Agar and transferred to phosphate buffered saline (pH 7.3). The content was centrifuged for 5 min. The supernatant was then discarded and the pellet was re-suspended in Tris-EDTA (TE) buffer. The suspension was heated using a Biosan (Latvia) thermomixer to 100°C degrees for 10 minutes. The boiled suspension was transferred directly onto ice and diluted by 1:10 in TE.

Molecular testing

The species-specific thermonuclease (*nuc*) gene for *S. pseudintermedius* as well as the 16S rRNR gene was tested by PCR using oligonucleotides described previously (Poulsen et al. 2003, Sasaki et al. 2010). The positive control strain for *S. pseudintermedius* (previously confirmed by sequencing analysis of the 16S rRNA gene) was obtained from the Laboratory of Antimicrobial and Biocide Resistance, Technical University of Lisbon. Oligonucleotides used for detection

of antimicrobial resistance and virulence genes are presented in Table 1.

Antimicrobial susceptibility

Antimicrobial susceptibility testing was performed using the broth microdilution method. Sensititre plates and the ARIS 2X automated system (Thermo Scientific) were used with the following antimicrobials: ceftriaxone, daptomycin, gatifloxacin, ciprofloxacin, clindamycin, erythromycin, gentamicin, levofloxacin, linezolid, oxacillin, penicillin, quinupristin/dalfopristin and rifampicin. Interpretation of results was carried out using the manufacturer's software (SWIN®) adapted to clinical breakpoints of the Clinical and Laboratory Standards Institute (CLSI). The quality control strain *S. aureus* ATCC 29213 was included in each assay for validation purposes.

PCR assays for antimicrobial and virulence genes

Detection of genes encoding antimicrobial resistance *(mecA, mecC, blaZ, tet*(K), *tet*(M), *erm*(A), *erm*(C), *msr*A/B, *aac(6')-Ie-aph(2'')-Ia, aph(3')-IIIa* and *dfrK*) was performed. Isolates were also tested for the *lukF*/*lukS* genes encoding leukocidin Luk-I and for the *siet* gene encoding exfoliative toxin. Annealing temperatures and oligonucleotides used are presented in Table 1. Positive control strains (previously confirmed by sequencing analysis) were obtained from the Laboratory of Antimicrobial and Biocide Resistance, Technical University of Lisbon.

Statistical analysis

Statistical analysis was performed using the „R 1.8.1" package (http://www.r-project.org/). Comparison between categorical variables was calculated using the chi-square test and Fisher's exact test. Results were considered statistically significant if $p<0.05$.

Results

One hundred and ninety two samples from 250 tested (76.8%) were positive for the presence of *Staphylococcus* species. The percentage was higher in non-treated animals (89.5%). Fifty-four isolates did not ferment mannitol on mannitol-salt agar, had a positive reaction with Microgen Staph Latex Kit and expressed a large double zone of haemolysis on sheep-blood agar. Thirty-two isolates were initially identified as *S. intermedius* using a biochemical identification system. Molecular typing using the species-specific *nuc* gene identified 51 isolates as *S. pseudintermedius* (26.6%) including those previously identified as *S. intermedius*. Fifteen samples (29.4%) from different dogs were able to grow on mannitol salt agar supplemented with 2 mg/L oxacillin. The *mecA* gene was detected in all of these isolates, all of them being resistant to oxacillin and identified as MRSP. The *mec*C gene was not detected. Ten MRSP isolates were isolated in two kennels (previously treated with fluoroquinolones and/or cephalosporins) breeding Yorkshire terriers, French Bulldogs and English Bulldogs (number of adult dogs in each kennel was 18 and 22 respectively).

Table 2 presents data on the distribution of *S. pseudintermedius* including MRSP isolates in dogs with different clinical infections, together with the number of isolates harbouring genes encoding production of Luk-I and *siet*.

The data presented in Table 2 demonstrate that *S. pseudintermedius* was isolated from dogs with different clinical infections, although the highest frequency of this species was detected in dogs with pyoderma. Ten MRSP isolates were isolated in two large breeding kennels from bitches with reproductive disorders.

Genes encoding production of Luk-I toxin were not associated with any of the clinical infections ($p>0.5$); however, the *siet* gene, responsible for the production of an exfoliative toxin, was significantly associated with isolates from skin infections ($p<0.01$). From all isolates, 68.6% had at least one gene responsible for the production of toxins.

Data on antimicrobial susceptibility of the isolates is presented in Table 3.

All *S. pseudintermedius* isolated strains were susceptible to vancomycin, daptomycin and linezolid. Two isolates (3.9%) were resistant to rifampicin. More frequent resistance was demonstrated to penicillin (94.1%), tetracycline (64.7%) and macrolides (68.7%). Resistance to fluoroquinolones was high and ranged from 25.5% (gatifloxacin) to 31.4% (ciprofloxacin). Genes encoding resistance to separate classes of antimicrobials were found in different numbers (Table 3). The most prevalent genes included *bla*Z, *aac(6')-Ie-aph(2'')-Ia*, *mecA*, and *tet*(M).

Discussion

Bacteria of the genus *Staphylococcus* are highly prevalent in clinical samples from small animals. In general, we found that 76.8% of the tested samples were positive although the prevalence was higher in

Table 2. Clinical infections associated with *S. pseudintermedius* including MRSP and presence of the genes encoding toxins.

Clinical disorder	Number of *S. pseudintermedius* isolates	Number of MRSP isolates	Genes encoding toxins		
			*luk*S	*luk*F	*siet*
Otitis	5	0	2	2	3
Pyoderma	24	4	6	6	22
Reproductive disorders	18	10	6	6	9
Other	4	1	1	1	1

Table 3. Antimicrobial susceptibility data and genes encoding resistance in *S. pseudintermedius* isolates (n=51).

Class of antimicrobials	Antimicrobial	Susceptibility[1], n (%)			Genes encoding resistance
		S	I	R	
Penicillins	Penicillin	3(5.9)	–	48(94.1)	*blaZ* (45)[3]
	Oxacillin	36(70.6)	–	15(29.4)	*mec*A (15)
Cephalosporins	Ceftriaxone	35(68.6)	3(5.9)	13(25.5)	
Tetracyclines	Tetracycline	18(35.3)	–	33(64.7)	*tet*(K) (6) *tet*(M) (14)
Macrolides, lincosamides and streptogramins	Erythromycin	14(27.5)	1(2.0)	36(70.6)	*erm*(A) (1)
	Clindamycin	14(27.5)	–	37(72.5)	*erm*(C) (3)
	Quinupristin/dalfopristin	48(94.1)	2(3.9)	1(2.0)	*msrA/B* (6)
DFR inhibitors	Trimethoprim	36(70.6)	–	15(29.4)	*dfrK* (8)
	Ciprofloxacin	35(68.7)	–	16(31.4)	nt
Fluoroquinolones	Gatifloxacin	38(74.5)	–	13(25.5)	nt
	Levofloxacin	36(70.6)	1(2.0)	14(27.5)	nt
Rifamycins	Rifampicin	49(96.1)	–	2(3.9)	nt
Lipopeptides	Daptomycin	51(100)	–	0(0)	nt
Glycopeptides	Vancomycin	51(100)	–	0(0)	nt
Aminoglycosides	Gentamicin	25(49.0)	7(13.7)	19(37.6)	*aac(6')-Ie-aph(2'')-Ia* (16) *aph(3')-IIIa* (11)
Oxazolidinones	Linezolid	51(100)	–	0(0)	nt

[1] S – susceptible; I – intermediate susceptible; R – resistant
[2] nt – not tested
[3] in parentheses – number of isolates harbouring the tested genes

non-treated animals (89.5%). Such data are consistent with data obtained by other authors (Griffeth 2008, Penna et al. 2010). We focused on *S. pseudintermedius* since it is the most prevalent species in dogs (Hauschild and Wójcik 2007, Penna et al. 2010, Bannoehr and Guardabassi 2012). Moreover, *S. pseudintermedius* is often reported as methicillin-resistant with co-resistance to different classes of antimicrobials other than beta-lactams (Perreten et al. 2010, Weese and Duijkeren 2010, Windahl et al. 2012). The number of *S. pseudintermedius* isolates (26.6%) revealed that this species is widely distributed in clinical samples from dogs although there are other species that are prevalent as well (data not presented).

There are different data about the prevalence of *S. pseudintermedius* described by other authors. For example, Feng et al. (2012) reported a prevalence of 18.3%, while Garbacz et al. (2013) described it to be 51.8.4% of tested animals. Data on the prevalence might depend on study design, identification methods, sampling, animal health status and other factors. It is known that classical biochemical tests for species identification are not always capable of identifying *S. pseudintermedius* (van Duijkeren et al. 2011). We found this to be true as well. Certain substrates (carbohydrates and amino acids) are weakly fermented and thus interpretation of results based on a colour index is subjective.

In our study *S. pseudintermedius* was isolated from dogs with different clinical infections, and thus we detected genes encoding for pathogenicity factors. We detected a high frequency (68.8%) of *luk* and/or *siet*

genes in *S. pseudintermedius* isolates in Lithuania. Interestingly, all isolates that harboured both *lukF* and *lukS* genes harboured the *siet* gene as well ($p<0.01$) but not *vice versa*. To our knowledge such a statistical relationship had not been detected before. Statistically reliable results were obtained when studying the association between the presence of *siet* gene in isolates from skin infections compared with isolates obtained from other types of infection. This agrees with the fact that the exfoliative toxin is a virulence factor of *S. pseudintermedius* involved in canine pyoderma. It is mainly found among isolates from skin infections (Lautz et al. 2006, Iyori et al. 2010). The *luk* genes were found in different *S. pseudintermedius* strains irrespective of the source of isolation. The Luk-I shows strong leucotoxicity towards various polymorphonuclear cells (Futagawa et al. 2004a). It might be responsible for invasion of the host by suppressing its cellular immunity and could be produced by different strains of *S. pseudintermedius*.

Data on antimicrobial susceptibility revealed that *S. pseudintermedius* most frequently has resistance to antimicrobials used in dogs including penicillins (resistance attributed to *bla*Z gene), tetracyclines (*tet*(K) and *tet*(M) genes) and macrolides (*erm*A and *erm*C genes).

The *erm*B gene was not detected. In fact, this gene is rarely isolated (1.9%) in MRSP isolates from Europe and North America collected previously (Perreten et al. 2010), except in Norway where resistance of *S. pseudintermedius* was attributed to the *erm*B gene (Norstrom et al. 2009). A high rate of resistance to fluoroquinolones (25.5-31.4%) was also recorded. Resistance mechanisms to fluoroquinolones of *S. pseudintermedius* are well described (Descloux et al. 2008). The high frequency of resistance to fluoroquinolones found here could be explained in that fluoroquinolones are frequently used to treat dog infections especially in cases with unsatisfactory clinical practice where broad-spectrum antimicrobials are selected for treatment without sending clinical material to a laboratory for diagnosis and antibiogram. Resistance of *S. pseudintermedius* isolates to gentamicin was also high (37.6%). The genes responsible for encoding resistance to aminoglycosides *aac(6')-Ie-aph(2")-Ia* and *aph(3')-IIIa* were detected here. The same genes were recently found in most isolates of enterococci isolated from diseased cows, pigs and poultry in Lithuania (Seputiene et al. 2012). Those genes encoding resistance to aminoglycosides were also found in *S. pseudintermedius* in other countries (Kadlec et al. 2010). It is interesting that resistant isolates to gentamicin harboured the *siet* gene as a rule ($p<0.01$).

In this study, 29.4% of *S. pseudintermedius* strains were identified as MRSP. Lithuania-specific data on methicillin resistant staphylococci isolated from animals is scarce – the first case of methicillin resistance in livestock was only reported in 2011, when MRSA ST398 strains were found and characterised in pigs (Ruzauskas et al. 2013). To the best of our knowledge, this study is the first Lithuanian study where MRSP strains were confirmed using molecular methods. This high frequency of MRSP in dogs could be explained as follows: first, only diseased animals were involved in the study and most of them were being treated or had been treated previously with an antimicrobial. Second, as proven before, the use of fluoroquinolones and cephalosporins might select for antimicrobial resistant bacteria (SAGAM 2009, van Duijkeren et al. 2011) and some of these dogs had been previously treated with some of these antimicrobial drugs. Finally, 10 MRSP isolates were isolated on two large breeding kennels from bitches with reproductive disorders in which the owners irregularly used antimicrobials, including enrofloxacin and/or cefovecin for "better reproductive performance". Such inappropriate use of antimicrobials possibly led to high resistance rates of MRSP in these kennels. Thus, breeding kennels might be a reservoir of MRSP strains and may pose a risk for spreading such strains during mating. There is no requirement for reporting MRSP or MRSA strains prevalent in kennels. Thus, other breeders have no information about the status of such animals. Attention should be paid to this problem since methicillin-resistant staphylococci pose a risk not only to animals but also to humans (Catry et al. 2010, Stegmann et al. 2010, van Duijkeren et al. 2011).

Our findings indicate that staphylococci including *S. pseudintermedius* are very common in clinical samples from diseased dogs. The prevalence of genes encoding toxins in *S. pseudintermedius* is high as also the resistance rates to some critically important antimicrobials such as beta-lactams and fluoroquinolones. Isolates remain susceptible only to those antimicrobials that are still banned from veterinary use (linezolid, vancomycin, and daptomycin). Such antibiotics should be reserved for humans and control of their use in kennels needs to be improved. Monitoring of antimicrobial resistance in kennels should be performed routinely and should include control options to avoid the spread of resistance.

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