Characterisation of methicillin-susceptible *Staphylococcus pseudintermedius* isolates from canine infections and determination of virulence factors using multiplex PCR

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ABSTRACT: *Staphylococcus pseudintermedius* is a genuine opportunistic pathogen of the skin, especially in canids. However, characterisation of virulence, antimicrobial resistance and genotypic variability in methicillin-susceptible *S. pseudintermedius* isolates has not been fully explored. In this study, coagulase-positive staphylococcal isolates collected from dogs of various breeds and ages suffering from dermatitis ($n = 70$), pyoderma ($n = 7$), and otitis ($n = 7$), from districts of Prague (Czech Republic) and surrounding areas, were characterised using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry, and repetitive sequence-based PCR fingerprinting. Susceptibility to antimicrobial agents was determined, virulence factor genes for leukocidin (*lukSF*-I), exfoliatins (*exi*, *expB*, and *siet*), enterotoxin C (sec_{canine}) and enterotoxin-related genes (*se-int* and *sel*) were detected using multiplex PCR and the genotypes of *S. pseudintermedius* isolates were determined using *Sma*I macrorestriction analysis. The majority of the staphylococcal isolates ($n = 84$) were identified as *S. pseudintermedius* ($n = 79$) and all of them were susceptible to methicillin/oxacillin (MSSP). About half of the strains ($n = 41$) were resistant to macrolide-lincosamide-streptogramin B antimicrobial agents and resistance was mediated in all but one of the strains by the *erm*(B) gene. The genes for *lukSF*-I, *siet*, *se-int*, and *sel* were detected in the majority of the MSSP strains (96.2%, 100%, 100%, and 73.4%, respectively). Investigated canine *S. pseudintermedius* isolates were highly heterogeneous, which prevented the correlation of any specific lineage to a particular infection, dog breed, or region of origin.

Keywords: *Staphylococcus pseudintermedius*; macrolide lincosamide-streptogramin B (MLS$_B$) resistance; genotyping; virulence genes

Staphylococcus pseudintermedius, a pathogen that infects dogs, is comparable to *Stapylococcus aureus* in humans. This analogy fits not only the phenotypic characteristics (e.g. similar colony morphology, pigment, haemolysis), but is also supported by the striking similarity in ecology/epidemiology (colonisation or infection of the skin and skin adnexa, vertical/horizontal transmission), expression of homologous virulence factors (cell wall-anchored proteins such as microbial surface components recognising adhesive matrix molecules; protein A; and enzymes, e.g. coagulase; secreted toxins, such as cytotoxins, exfoliative toxins, or superantigens) and pathogenicity (skin infections or invasive infections). These two coagulase-positive species probably evolved separately through adaptation

to their respective hosts. Even today, differentiating *S. pseudintermedius* and *S. aureus* can pose a problem for diagnostic laboratories (Weese 2013).

A large proportion of healthy dogs (37%) and the majority of infected dogs (87%) are colonised by *S. pseudintermedius* (Fazakerley et al. 2010), which also has the potential to be an agent of zoonotic infections (Borjesson et al. 2015).

In genotyping of *S. pseudintermedius*, macrorestriction analysis resolved by pulsed-field gel electrophoresis (PFGE) has higher discriminatory power than staphylococcal protein A (*spa*) typing (Moodley et al. 2013) or multilocus sequence typing (Solyman et al. 2013) and, therefore, is currently considered as the most suitable method for the typing of a large collection of isolates. Even though *spa* typing yields more stable and reproducible results and is thus superior to PFGE DNA macrorestriction analysis in the determination of clonality, it is not as effective as the latter method in typing methicillin-susceptible isolates of which more than 50% are non-typeable (Bannoehr and Guardabassi 2012).

Only a few local studies have been performed on *S. pseudintermedius* isolates from Eastern and Central Europe, and infectious agents in household pets and the use of antimicrobial agents in these animals require more focus. The goal of the present study was to characterise *S. pseudintermedius* isolates recovered from dogs varying in breed, sex, and age and coming from various districts of the City of Prague. We here describe a newly developed multiplex PCR assay for determination of virulence factors in such isolates.

MATERIAL AND METHODS

Sample collection and bacterial isolates. The dog owners presented their animals to our laboratory for identification of specific infectious agents causing secondary dermatitis or pyoderma. Samples from the affected lesions were collected in 2005 using dacron swabs, stored until inoculated onto Columbia blood agar with 5% Sheep Blood (Oxoid, UK) and cultured in aerobic atmosphere at 37 °C. Single canine coagulase-positive staphylococcal isolates (n = 84) were cultured from dogs of various breeds and ages suffering from dermatitis (n = 70), pyoderma (n = 7), and otitis (n = 7) to diagnose the specific agents of canine skin or ear infections. The owners of the dogs lived in nine specified Prague City districts (n = 36), an unknown Prague district

(n = 40), and surrounding areas (n = 8). Samples were collected from dogs of different breeds (n = 43) and four crossbred dogs. The reference strains were obtained from the Czech Collection of Microorganisms (Masaryk University, Brno).

Phenotypic identification. Identification of all isolates was performed using a matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry MALDI-TOF MS system (Microflex™ LT/RF, database 5626, Bruker, Bremen, Germany).

Genotypic identification. Genotypic screening of the isolates was performed using rep-PCR fingerprinting with the $(GTG)_5$ primer, reported as a suitable tool for the identification of *Staphylococcus* spp. (Svec et al. 2010). The resulting fingerprints were processed using BioNumerics v. 7.5 software (Applied-Maths, Kortrijk, Belgium) and compared to the in-house $(GTG)_5$-PCR fingerprints database (Svec et al. 2010).

Antimicrobial agent susceptibility. Tests for susceptibility to antimicrobial agents were performed using Mueller-Hinton agar (CM0337, Oxoid, UK) and disks (Oxoid, UK) of oxacillin, cefoxitin, amoxicillin/clavulanic acid, cefalotin, ofloxacin, mupirocin, erythromycin and clindamycin and the disk diffusion method results were interpreted using the criteria of EUCAST (http://www.eucast.org) and strain ATCC 29213 *S. aureus* as a control. Since a large proportion of the isolates were resistant to macrolide-lincosamide-streptogramin B (MLS_B) antimicrobial agents (erythromycin, clindamycin) the presence of the *erm*(A), *erm*(B), *erm*(C), and *msr*(A) genes was determined in the resistant isolates (Lina et al. 1999).

Macrorestriction analysis of DNA. Electrophoresis was performed using the CHEF DR II system (Bio-Rad, California, USA). All *S. pseudintermedius* isolates (n = 79) were typed using the *Sma*I restriction endonuclease and PFGE in order to assess their population structure. Bacterial DNA was harvested from cultures grown on nutrient agar plates and agarose plugs were prepared according to the manufacturer's recommendations. The *Sma*I macrorestriction profiles of the isolates were evaluated by visual inspection and the dendrogram was constructed using BioNumerics v. 6.5 software (Applied Maths, Kortrijk, Belgium) with the Dice similarity coefficient and unweighted pair group method with arithmetic mean (UPGMA) clustering. An optimisation of 1.5% and a position tolerance of 4% were allowed in the cluster analysis and calculation of the dendrogram.

Detection of virulence factor genes. Multiplex PCR assays I and II to detect virulence genes were carried out using a Multiplex PCR kit (Qiagen, Hilden, Germany) in a final volume of 25 μl, which contained 2 μl aliquots of the crude boiled-cell lysates. The primers used for the PCR assays were designed specifically for this study as detailed in Table 1. Primers targeting the *S. intermedius* group (SIG) thermonuclease gene (*sI nuc*) were used as an internal positive control (Baron et al. 2004). The PCR assays were performed using a T-Gradient thermal cycler (Biometra, Goettingen, Germany) set to the following parameters: 35 cycles of denaturation (94 °C, 30 s), annealing (53 °C, 30 s), and extension (72 °C, 60 s). The efficiency of the PCR assay was evaluated by performing reactions using 10-fold serial dilutions of purified genomic DNA.

RESULTS

Phenotypic identification

Using MALDI-TOF MS we identified the majority ($n = 79$; 94%) of the staphylococcal isolates ($n = 84$) as *S. pseudintermedius* and only sporadic isolates as *Staphylococcus schleiferi* ($n = 3$) and *S. aureus* ($n = 2$). Species level identification revealed minor differences between the results obtained for the top score corresponding to *S. pseudintermedius*, and the second match *Staphylococcus intermedius*. Therefore, rep-PCR genotypic identification was carried out. *S. aureus* was identified as described previously (Stepan et al. 2001).

Genotypic identification using rep-PCR with the (GTG)$_5$ primer

Numerical analysis of rep-PCR fingerprints confirmed the identification results obtained by MALDI-TOF MS analysis. All *S. pseudintermedius* isolates revealed visually similar fingerprints matching with the database fingerprint entries for the *S. pseudintermedius* reference strains and were grouped in a single cluster (Figure 1). Similarly, *S. schleiferi* and *S. aureus* isolates matched the reference fingerprints representing these taxa. Moreover, the rep-PCR analysis of *S. schleiferi* strains assigned all three isolates as representatives of *S. schleiferi* subsp. *coagulans*.

Table 1. Primers used for multiplex PCR assays targeting toxin-related genes

| Primer	Sequence	Product (bp)	Primer concentration	GenBank accession No.	Target gene
Multiplex PCR I					
lukF-sig_F lukS-sig_R	AGCCAATAGTTTTATTATCTGTG AAATTAAAGTAAAGGGGCAT	595	100nM	X79188	*lukS*-I and *lukF*-I gama haemolysin components
exi_F exi_R	AGTAACAAACTATCACATAGCG TTAACAGGTTATAACGTCCCC	455	40nM	AB489850	*exi* gene for exfoliative toxin
expb_F expb_R	AAATTATTTTCACTCCAGCTT CATGTATACCTATTAGTTCCCC	381	40nM	AB569087	*expB* gene for exfoliative toxin
se_sig_F se_sig_R	CATTTGAAATACAAGCCGAC TGATTAAAATTATTACCCGGTG	199	25nM	AB116378	enterotoxin-related gene *se-in*
Multiplex PCR II					
et_sig_F et_sig_R	GATAAAATTTATGCGGGTCCT ATTACTTTCTTCAGGGTCGAA	417	50nM	AB099710	*siet* gene for exfoliative toxin
sel_sig_F sel_sig_R	TCTTTTTGTGGTGCCTGA ATGTCTTTATCATTATCCCCA	306	100nM	CP002439 and CP002478	conserved region of genes SPSINT_0099 and SPSE_2371 for superantigen like proteins
sec_can_F sec_can_R	CAGCAACTAAAGTTAAGTCTG ACATACAAGTTTTACCACCT	234	25nM	U91526	*sec*$_{canine}$ gene for type C enterotoxin
SInuc1 SInuc2	CAATGGAGATGGCCCTTTTA AGCGTACACGTTCATCTTG	125	50nM	X67678	*nuc* gene for *S. intermedius* group thermonuclease
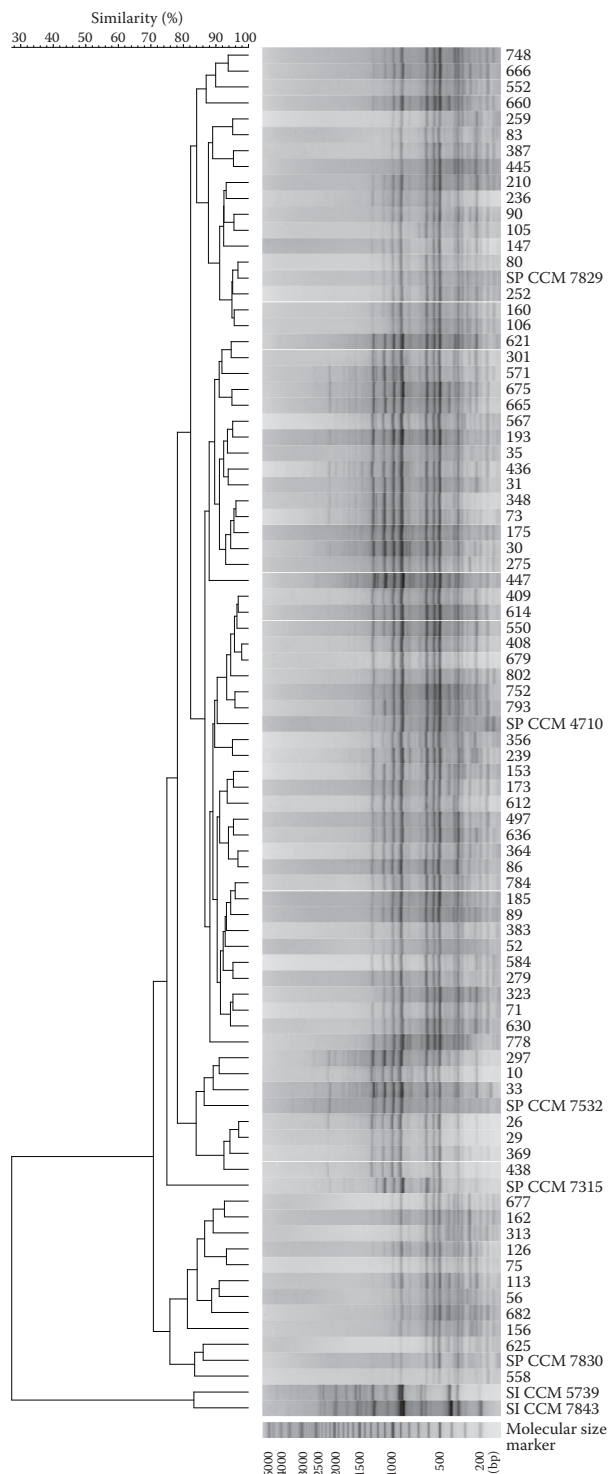
Figure 1. Dendrogram based on rep-PCR fingerprints obtained from *S. pseudintermedius* (SP) canine isolates and reference strains of *S. intermedius* (SI) and *S. pseudintermedius*. The dendrogram was calculated using Pearson's correlation coefficients with the UPGMA clustering method (*r*, expressed as percentage similarity values)

Antimicrobial agent resistance and detection of MLS$_B$ determinants

All of the *S. pseudintermedius* isolates tested were susceptible to methicillin/oxacillin (MSSP), amoxicillin/clavulanic acid, cefalotin, and ofloxacin; however, half of the isolates (*n* = 41; 51%) were constitutively resistant to erythromycin and clindamycin. Testing for the presence of the *erm*(A), *erm*(B), *erm*(C) and *msr*(A) genes revealed that, in all but one of the isolates, resistance was mediated by *erm*(B). Only sporadic isolates (*n* = 4) were resistant to mupirocin.

Macrorestriction analysis of DNA

The macrorestriction patterns of 77 typeable isolates of *S. pseudintermedius* revealed 46 clusters when the similarity threshold value of 93% proposed by Paul et al. (2012) was used (Figure 2). Closely related isolates from the present study were not breed-related. Each fingerprint cluster in the present study included from one to six *S. pseudintermedius* strains. All isolates in our study shared a similarity of higher than 72%.

Frequency and distribution of virulence factors

Seven virulence genes were found in this study: the enterotoxin-related gene *se-int* and the *siet* gene for exfoliative toxin were detected in all *S. pseudintermedius* isolates. The genes for two-component leukotoxin (*lukS*-I and *lukF*-I) were also frequently detected (*n* = 76; 96.2%). The majority of the analysed isolates (*n* = 58; 73.4%) carried another enterotoxin-related gene, *sel*. The remaining genes *exi*, *sec*$_{canine}$, and *expB* were detected in 18 (22.8%), 13 (16.5%), and six (7.6%) isolates, respectively. These genes were found in different combinations, defining 12 different toxin-related genotypes (Figure 3 and Table 2). The combination of *lukSF*-I, *se-int*, *siet*, and *sel* was the most common and was detected in 54.4% of the isolates. The distribution of the virulence genes in relation to clinical diagnosis was not statistically evaluated due to the relatively high number of different combinations of toxin genes and the small numbers of isolates from pyoderma and otitis cases. Genotyping revealed that more
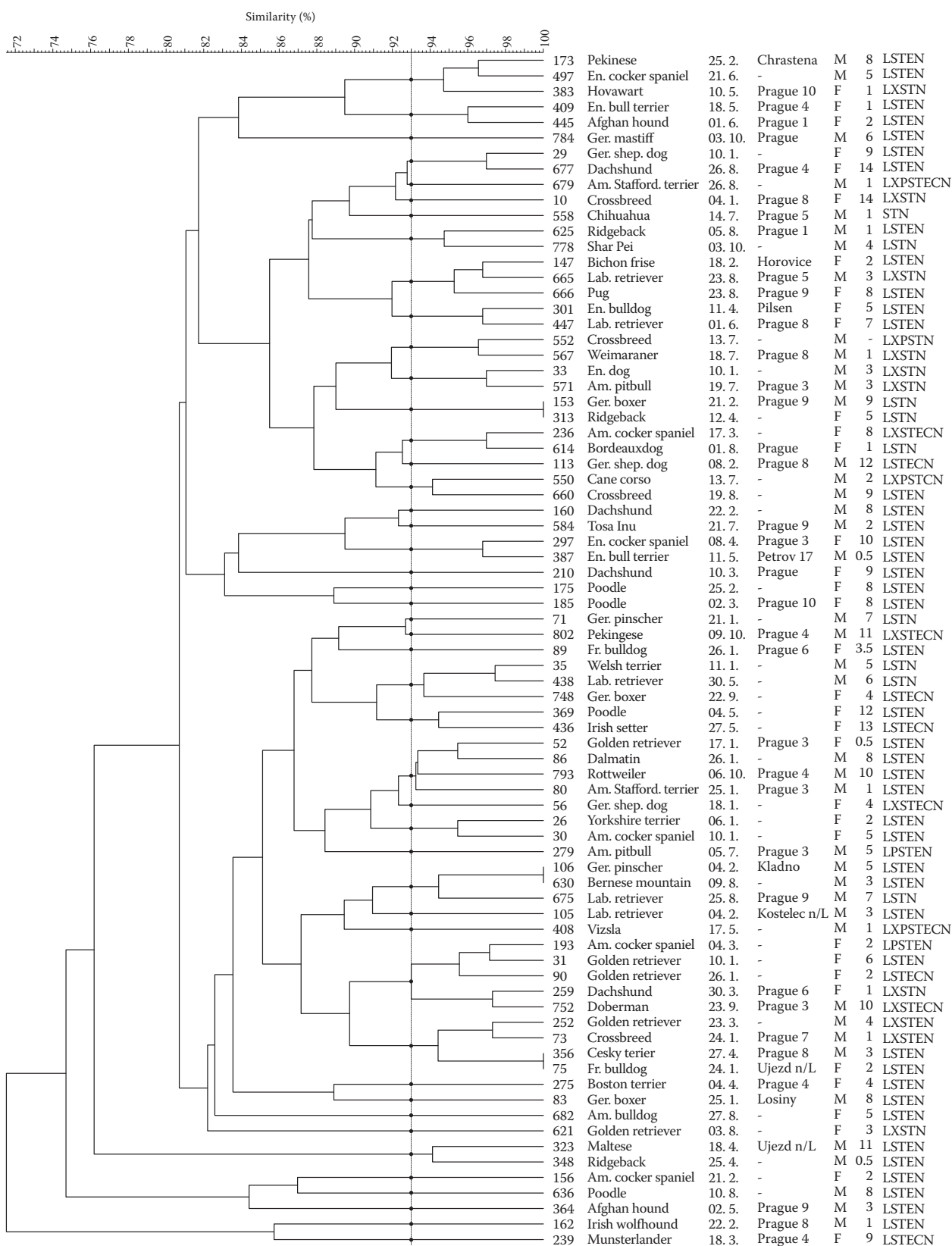
Figure 2. Dendrogram illustrating the similarity (based on the Dice coefficients and UPGMA clustering) of *Sma*I macrorestriction profiles (PFGE) of *S. pseudintermedius* canine isolates. Symbols denote from left to the right: number of isolate, dog breed, collection date (2005), origin, sex, age (years), and virulence genotype (C, *sec_canine*; E, *sel*; L, *lukSF*-I; N, *sI nuc*; P, *expB*; S, *se-int*; T, *siet*; X, *exi*)
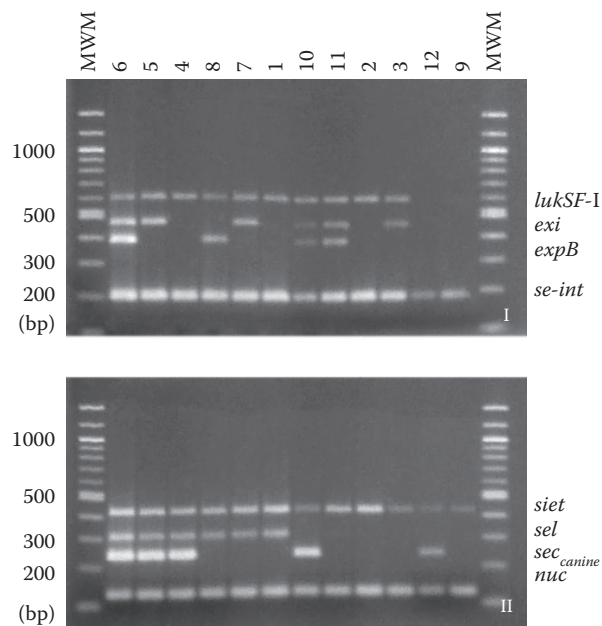
Figure 3. Representative agarose gel electrophoretic patterns revealing the different toxin-related gene profiles generated using multiplex PCR assays I and II in *S. pseudintermedius* strains. Profile numbers correspond to Table 2. MWM, Molecular Weight Marker (100 bp DNA Ladder, New England BioLabs)

DISCUSSION

MALDI-TOF MS analysis, recommended by other authors (Decristophoris et al. 2011) for identification of the SIG species, correctly identified all *S. pseudintermedius* isolates and two other staphylococcal species in this study. Interestingly, the proportion of *S. aureus* (*n* = 2; 2.5%) and *S. schleiferi* subsp. *coagulans* (*n* = 3; 3.8%) differs from that reported by Slettemeas et al. 2010 (0.5%) and Chanchaithong and Prapasarakul 2011 (0.9%) for *S. aureus* strains and also from that described by Slettemeas et al. 2010 (7%) and Kawakami et al. 2010 (10.5%) for *S. schleiferi* subsp. *coagulans* strains.

The rep-PCR fingerprinting method proved to be useful for the identification of all isolates; its advantage over MALDI-TOF MS is that it allowed for the unambiguous differentiation between *S. pseudintermedius* and *S. intermedius* and final identification of *S. schleiferi* subsp. *coagulans* to the subspecies level.

The study isolates were collected in 2005; therefore, it is not surprising that no MRSP isolates were found, as methicillin-resistant *S. pseudintermedius* (MRSP) emerged in Europe in 2005–2006 (Loeffler et al. 2007; Schwarz et al. 2008; Ruscher et al. 2009; Ruscher et al. 2010). The resistance to MLS$_B$ antimicrobial agents in half of the *S. pseudintermedius* isolates revealed in this study is close to the 48.7% reported by Garbacz et al. (2011), and the 42% and approximately 38% methicillin-susceptible isolates found by Kawakami et al. (2010) and Haenni et al. (2014), respectively.

than half of the *S. pseudintermedius* isolates (*n* = 58) carried four virulence genes encoding leukotoxin (*lukSF*-I), enterotoxin (*se-int*), exfoliative toxin (*siet*), and superantigen-like protein (*sel*). Thus, it can be presumed that these four genes play a major role in pathogenesis. Other virulence profiles could only be seen in a few isolates.

Table 2. Prevalence of toxin-related genotypes in *Staphylococcus pseudintermedius* isolates

Profile	No. of genes	No. (%) of isolates (*n* = 79)	No. (%) of isolates by clinical sample			Gene combination						
			dermatitis (*n* = 66)	pyoderma (*n* = 6)	otitis (*n* = 7)	*lukSF*-I	*exi*	*expB*	*se-int*	*siet*	*sel*	*sec$_{canine}$*
1	4	43 (54.4)	38 (57.6)	1 (16.7)	4 (57.1)	+	–	–	+	+	+	–
2	3	8 (10.1)	8 (12.1)	–	–	+	–	–	+	+	–	–
3	4	8 (10.1)	4 (6.1)	3 (50.0)	1 (14.3)	+	+	–	+	+	–	–
4	5	5 (6.3)	5 (7.6)	–	–	+	–	–	+	+	+	+
5	6	4 (5.1)	3 (4.5)	–	1 (14.3)	+	+	–	+	+	+	+
6	7	2 (2.5)	2 (3.0)	–	–	+	+	+	+	+	+	+
7	5	2 (2.5)	2 (3.0)	–	–	+	+	–	+	+	+	–
8	6	2 (2.5)	–	2 (33.3)	–	+	–	+	+	+	+	–
9	2	2 (2.5)	2 (3.0)	–	–	–	–	–	+	+	–	–
10	5	1 (1.3)	–	–	1 (14.3)	+	+	+	+	+	–	+
11	5	1 (1.3)	1 (1.5)	–	–	+	+	+	+	+	–	–
12	3	1 (1.3)	1 (1.5)	–	–	–	–	–	+	+	–	+

Substantially lower rates of MLS_B resistance, i.e. 16.7% and 27.7%, were reported by Onuma et al. (2012) in dogs with pyoderma in two periods.

The constitutive resistance of all MLS_B-resistant isolates was mediated almost exclusively by the *erm*(B) gene, as reported also by others (Kadlec and Schwarz 2012). The mechanism of resistance to MLS_B antimicrobial agents could not be identified in one of the resistant isolates, even when tested for additional resistance determinants (*erm*(A), *erm*(C), and *msr*(A)).

The heterogenic pulsotypes of the isolates fully correspond with the literature data indicating high genetic diversity of methicillin-susceptible *S. pseudintermedius* (MSSP) isolates (Norstrom et al. 2009) compared with that of clonally related methicillin-resistant isolates of *S. pseudintermedius* (MRSP; Hesselbarth et al. 1994; Bannoehr et al. 2007; Fazakerley et al. 2010; Perreten et al. 2010; Garbacz et al. 2011; Gomez-Sanz et al. 2013; Couto et al. 2014). However, some authors failed to observe any diversity of *S. pseudintermedius* strains in healthy and diseased dogs (Sasaki et al. 2005).

A newly designed PCR assay, based on two multiplex reactions, confirmed the presence of virulence factor genes in all *S. pseudintermedius* isolates and would be suitable for use in diagnostic laboratories. The prevalence of the *siet* gene (100%) was higher than described by others, e.g. 46.6% (Lautz et al. 2006), but almost equal to the percentage reported by Yoon et al. (2010). The occurrence of the *exi* gene (22.8%) was similar to that observed by Futagawa-Saito et al. (2009) and the prevalence of the *expB* gene (7.6%) was lower than the 23% indicated by Iyori et al. (2010). The prevalence of the enterotoxin-related gene sec_{canine} (16.5%) was close to the 12.6% reported by Becker et al. (2001) but lower than the 24% described by Yoon et al. (2010) and other authors who confirmed its presence in all tested isolates (Futagawa-Saito et al. 2004). The enterotoxin production rate was previously described to be significantly higher in isolates from diseased dogs in comparison with healthy dogs (Sasaki et al. 2005), but not in all studies (Tanabe et al. 2013). The role of *S. pseudintermedius* enterotoxin-related toxins in the pathogenesis of dog skin infections is still not fully clarified. However, strain superantigenicity, ability to induce cytokine production, pyrogenicity, lymphocyte proliferation and immunosuppression could potentiate the skin inflammation process (Hendricks et al. 2002).

In conclusion, the rep-PCR method employed here was superior to MALDI-TOF MS in its ability to identify canine coagulase-positive staphylococci to the subspecies level. Constitutive resistance to MLS_B antimicrobial agents (erythromycin, clindamycin) in half of the *S. pseudintermedius* isolates (41 from a total of 79) was determined by the presence of the *ermB* gene in all but one isolate. Moreover, macrorestriction analysis of DNA of the *S. pseudintermedius* isolates using pulsed field gel electrophoresis revealed their high genotypic heterogeneity. A newly designed PCR assay was used to detect numerous virulence factor genes, of which four – encoding leukotoxin (*lukSF*-I), enterotoxin (*se-int*), exfoliative toxin (*siet*), and superantigen-like protein (*sel*) – were detected together in the majority of the isolates ($n = 58$), suggesting a significant role for these factors in the pathogenesis of canine skin infections.

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