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Original Article
Özgün Araştırma

Frequency of *Staphylococcus pseudintermedius* in canine skin infections and antibiotic resistance profiles of the recovered isolates

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Abstract: In this study, 61 *Staphylococcus pseudintermedius* strains were isolated from 77 dogs with skin infections. Antimicrobial resistance to commonly used antibiotics was evaluated by the disc diffusion method. The presence of the *blaZ* and *mecA* genes responsible respectively for penicillin and methicillin resistance was investigated by PCR. The *S. pseudintermedius* isolates were highly resistant to tetracycline (47.5%) and penicillin (40.9%) while all isolates were susceptible to amikacin (100%). All 18 methicillin-resistant *S. pseudintermedius* (MRSP) strains were positive for the *mecA* gene while the 25 *S. pseudintermedius* strain isolates with the *blaZ* gene were also resistant to penicillin phenotypically. Of the 18 MRSP isolates, 14 *S. pseudintermedius* strains were resistant to three or more antibiotics, indicating that these strains showed multiple drug resistance (MDR). This report confirms previous findings that *S. pseudintermedius* is the most frequently isolated bacteria from dog skin infections. It can also be concluded that amikacin is a useful agent for treating *S. pseudintermedius* infections since all the *S. pseudintermedius* strains tested in this study were susceptible to amikacin.

Keywords: Dogs, methicillin resistance, *staphylococcus pseudintermedius*

Köpek deri enfeksiyonlarında *Staphylococcus pseudintermedius* sıklığı ve elde edilen izolatların antibiyotik direnç profilleri

Özet: Bu çalışmada, deri enfeksiyonu olan 77 köpekten 61 *Staphylococcus pseudintermedius* suşu izole edilmiştir. Yaygın olarak kullanılan antibiyotiklere karşı antimikrobiyal direnç, disk difüzyon yöntemi ile değerlendirildi. Penisilin ve metisilin direncinden sorumlu sırasıyla *blaZ* ve *mecA* genlerinin varlığı PCR ile araştırıldı. *S. pseudintermedius* izolatları tetrasiklin (% 47.5) ve penisiline (% 40.9) yüksek direnç gösterirken, tüm izolatlar amikasine (% 100) duyarlıydı. 18 metisiline dirençli *S. pseudintermedius* (MRSP) suşunun tümü *mecA* geni için pozitif iken *blaZ* genine sahip 25 *S. pseudintermedius* suşu izolatı da fenotipik olarak penisiline dirençliydi. 18 MRSP izolatından 14 *S. pseudintermedius* suşu, üç veya daha fazla antibiyotiğe dirençliydi, bu da bu suşların çoklu ilaç direnci (MDR) gösterdiğini ortaya koydu. Bu rapor, *S. pseudintermedius*'un köpek deri enfeksiyonlarından en sık izole edilen bakteri olduğuna dair önceki bulguları doğrulamaktadır. Bu çalışmada test edilen tüm *S. pseudintermedius* suşları amikasine duyarlı olduğundan, amikasinin *S. pseudintermedius* enfeksiyonlarının tedavisinde yararlı bir ajan olduğu sonucuna varılabilir.

Anahtar kelimeler: Köpek, metisilin direnci, *staphylococcus pseudintermedius*

Introduction

Staphylococcus pseudintermedius is a coagulase-positive staphylococcal species described in 2005 as a distinct species from *Staphylococcus intermedius* (Devriese et al. 2005). It is classified in the *Staphylococcus intermedius* group (SIG), which comprises *S. pseudintermedis, S. intermedius* and *S. delphini* (Devriese et al. 2005; Sasaki et al. 2007a; Sasaki et al. 2007b).

S. pseudintermedius is an opportunistic pathogen that colonizes the skin and mucosal surfaces in cats and dogs (Devriese et al. 2005; Rubin and Chirino-Trejo 2011; Bannoehr and Guardabassi 2012). *S. pseudintermedius* also causes clinical infections, such as canine pyoderma, otitis externa, and nosocomial infections (Ball et al. 2008; van Duijkeren et al. 2011). *S. pseudintermedius* causes more than 90% of canine pyoderma cases (Kawakami et al. 2010; Yoon et al. 2010). Although this organism is associated with skin infections in dogs, human cases have also been described, especially among dog owners after contact with them (Van Hoovels et al. 2006; Chuang et al. 2010; Stegmann et al. 2010; Savini et al. 2013; Somayaji et al. 2016). Since the first report of human transmission of this pathogen, it has been accepted as a critical public health concern (Van Hoovels et al. 2006).

Since *S. intermedius, S. delphini* and *S. pseudintermedius* resemble each other phenotypically, classical biochemical tests are neither reliable nor sufficient for discriminating *Staphylococcus* intermedius

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group (SIG) members (Sasaki et al. 2007b; Bond and Loeffler 2012). Many methods used to discriminate *S. pseudintermedius* from other SIG members, such as restriction with certain enzymes of the housekeeping *pta* gene and *nuc* gene by PCR-restriction fragment length polymorphism (RFLP), the *hsp60* gene, 16S rDNA sequences, and the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (El Zubeir et al. 2007; Sasaki et al. 2007b; Bannoehr et al. 2009). The sequence analysis of the RNA polymerase B (*rpoB)* gene has been used to discriminate staphylococcal species and subspecies. This method is also recommended by the Clinical and Laboratory Standards Institute (CLSI) for identifying *S. pseudintermedius* (Mellmann et al. 2006; Petti and Clinical and Laboratory Standards Institute 2008).

Antibiotic resistance is a great challenge in treating both human and animal infections worldwide (Bannoehr and Guardabassi 2012). According to the literature, *S. pseudintermedius* remains susceptible to antibiotics, although there has been a dramatic increase in resistance to methicillin since 2006 (van Duijkeren et al. 2011; Moodley et al. 2014). Detection of methicillin resistance in *S. pseudintermedius* (MRSP) by the determination of the *mecA* gene has become a "gold standard" method (Geha et al. 1994; Chambers 1997). This gene encodes a penicillin-binding protein 2a/2' (PBP2a/PBP2'), which has a lower affinity to all beta-lactam group antibiotics. It, therefore, causes resistance to all beta-lactam and beta-lactam derivative antibiotics (Felten et al. 2002; Velasco et al. 2005; Bosgelmez-Tinaz et al. 2006; Zhu et al. 2006; Perez et al. 2007).

Beside this, beta-lactam antibiotics such as penicillin have been used in staphylococcal infections in both humans and animals. Penicillin-resistant staphylococci are the most commonly isolated strains reported in dogs and cats (Fuda et al. 2005; Malik et al. 2007). Penicillin resistance in staphylococci is mainly caused by the *blaZ* gene, which is similar to the *mecA* gene. It encodes beta-lactamases and is considered to mediate methicillin resistance (Zscheck and Murray 1993; Rosato et al. 2003). Recently, there have been reports of multi-antimicrobial resistance in *S. pseudintermedius* (Bannoehr and Guardabassi 2012; De Lucia et al. 2012; Murugaiyan et al. 2014).

The present study aimed to determine the presence of MRSP strains and the multiple drug resistance (MDR) profile of *S. pseudintermedius* strains isolated from skin infections of dogs.

Materials and Methods

Isolation and identification

This study used 68 staphylococcal isolates obtained from swab samples taken from 77 dogs diagnosed with skin infections in an animal hospital in Ankara University and submitted to the Department of Microbiology between 2014 and 2018. All the staphylococcal isolates were subjected to standard microbiological procedures. After incubation on blood agar containing 5-7% ovine blood at 37°C for 24h, Gram staining, catalase test, DNAse test, and coagulase test were performed. Microbact™ Staph 12S system (Staphylococcal 12S Identification System, MB1561, Oxoid) was used for further phenotypic identification.

Molecular identification of S. pseudintermedius isolates

For this purpose, the sequence analysis of the *rpoB* gene was performed (CLSI 2018). Briefly, the total volume of 25 µl PCR mix consisted of 3 mM $MgCl_2$ (Thermo Fischer Scientific, USA), 0.2 mM dNTPs (10 mM dNTP mix; Thermo Fischer Scientific, USA), 0.2 µM of each primer, 2.5 µl PCR reaction buffer, 2U Taq DNA polymerase (Thermo Fischer Scientific, USA) and 2 µl template DNA. The PCR amplification conditions were as follows: 94°C for 3 min, 30 cycles of 94°C for 1 min, 58°C for 30 s, 72°C for 1 min, and 72°C for 7 min. *S. pseudintermedius* strain obtained from Dr. Ken Kikuchi (Department of Infection Control Science, Faculty of Medicine Juntendo University, Tokyo, Japan) was used as a positive control in all phenotypic and genotypic tests.

After amplification, the PCR products were purified using a QIAquick PCR purification kit (Qiagen). For the sequence analysis, BigDye Direct Cycle Sequencing Kit (Applied Biosystems) was used according to the manufacturer's instructions. Sephadex G-50 (Sigma-Aldrich) was used for purifying the amplicons while the sequence analysis was performed in an Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems). CLC Main Workbench software version 7 was used for analyzing sequences. The primers used for the amplification and sequencing of the *rpoB* gene are shown in Table 1 (Drancourt et al. 2004).

Table 1. Primers used for amplification and sequencing of the *rpoB* gene.

	Primer Sequences	Product	References
31F 830R	F 5'- GCCTTAGGACCTGGTGGTTT-3' R 5'- GTTGTAACCTTCCAWGTCAT-3'	800 bp	Drancourt, 2004

Antimicrobial Susceptibility Tests

The antimicrobial susceptibility test was performed by disc diffusion following the recommendations of CLSI. Discs known to be used in veterinary medicine worldwide were chosen and used as follows: amikacin (AK: 30 µg); amoxicillin-clavulanic acid (AMC: 30 µg); chloramphenicol (C: 30 µg); ciprofloxacin (CIP: 5 µg); erythromycin (E: 15 µg); gentamicin (GM: 10 µg); kanamycin (K: 30 µg); neomycin (N: 10 µg); penicillin (P: 10 µg); streptomycin (S: 10 µg); tetracycline (TE: 30 µg); trimethoprim-sulfamethoxazole (SXT: 5 µg). For the methicillin resistance, oxacillin (OX: 1 µg) disc was used according to CLSI guidelines.

PCR Amplification of Methicillin and Penicillin Resistance Genes

Methicillin and penicillin resistance genes were investigated by detecting the *mecA* and *blaZ* genes described by Strommenger et al. (2003) and Martineau et al. (2000), respectively. PCR amplification was performed containing 0.2 µM of each primer, 0.2 mM dNTPs (10 mM dNTP mix; Thermo Fisher Scientific, USA), 3 mM MgCl2 (Thermo Fisher Scientific, USA), 2.5 µL PCR reaction buffer, 2U Taq DNA polymerase (Thermo Fisher Scientific; EP0402), 2 µL of DNA as a template and nuclease-free water to produce a final volume of 25 µl. PCR conditions were as follows: strand separation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 7 min at 72°C for further strand extension.

Results

A total of 61 isolates from 68 staphylococcal isolates were identified as *S. pseudintermedius* by both biochemical and sequence analysis of the *rpoB* gene while 7 isolates of 68 staphylococcal isolates were identified as *Staphylococcus delphini*.

The antimicrobial susceptibility of the 61 isolates is shown in Table 2. The lowest antibiotic resistance was observed for amikacin, gentamycin, and chloramphenicol whereas the highest resistance was observed for tetracycline and penicillin. The isolates that were resistant to at least three antibiotics were considered MDR isolates. According to CLSI guidelines, 25 *S. pseudintermedius* strains showed an MDR phenotype, 18 *S. pseudintermedius* strains had an MRSP phenotype, while 14 *S. pseudintermedius* strains had both MDR and MRSP phenotypes (Table 3). Beside this, 25 *S. pseudintermedius* strains were resistant to penicillin.

The *mecA* and *blaZ* genes were also observed in 18 and 25 strains, respectively, which indicates that these isolates were *mecA*-mediated MRSP isolates (Bemis et al. 2006). In addition, all MRSP isolates showed high antibiotic resistance (77.7%), except for amikacin. All the penicillin-resistant strains except for one showed an MDR phenotype.

Table 2. Antimicrobial susceptibility of the 61 *S. pseudintermedius* isolates.

Antimicrobial agent	Number of isolates (%)		
	Susceptible	Intermediate	Resistant
Amikacin	61 (100)	0 (0)	0 (0)
Amoxicillin-clavulanic acid	39 (63.9)	0 (0)	22 (36)
Chloramphenicol	50 (81.9)	0 (0)	11 (18)
Ciprofloxacin	47 (77)	0 (0)	14 (22.9)
Erythromycin	35 (57.3)	4 (6.5)	22 (36)
Gentamicin	50 (81.9)	4 (6.5)	7 (11.4)
Kanamycin	38 (62.2)	0 (0)	23 (37.7)
Neomycin	29 (47.5)	11 (18)	21 (34.4)
Oxacillin	37 (60.6)	6 (9.8)	18 (29.5)
Penicillin	36 (59)	0 (0)	25 (40.9)
Streptomycin	39 (63.9)	0 (0)	22 (36)
Tetracycline	32 (52.4)	0 (0)	29 (47.5)
Trimethoprim-sulfamethoxazole	47 (77)	0 (0)	14 (22.9)

Table 3. Antimicrobial susceptibility of the 18 MRSP isolates.

Antimicrobial agent	Number of isolates		
	Susceptible	Intermediate	Resistant
Amikacin	18	0	0
Amoxicillin-clavulanic acid	8	0	10
Chloramphenicol	15	0	3
Ciprofloxacin	4	0	14
Erythromycin	5	0	13
Gentamicin	14	0	4
Kanamycin	4	0	14
Neomycin	7	0	11
Penicillin	4	0	14
Streptomycin	4	0	13
Tetracycline	5	0	14
Trimethoprim-sulfamethoxazole	4	0	14

Discussion

In this study, the isolation rate of *S. pseudintermedius* was high for all staphylococcal isolates. Of the 68 staphylococcal isolates, 61 were *S. pseudintermedius*, of which 25 were MDR and 18 were MRSP. On the other hand, 18 strains of the isolates were *mecA* positive while 25 were *blaZ* positive by PCR.

S. pseudintermedius was detected in 89.7% of the dogs with skin infections by *rpoB* gene sequencing. This high isolation rate was also observed in previous studies (Kawakami et al. 2010; Bannoehr and Guardabassi 2012). Onuma et al. (2012) reported *S. pseudintermedius* isolation rates of 76% in dogs with pyoderma while Saputra et al. (2017) reported 70%. Although the comparison between different studies cannot be made easily due to the differences in the geographical region, sample type, and identification methods, these results indicate that *S. pseudintermedius* is the major causative agent of canine skin infections. In Turkey, the rate of *S. pseudintermedius* based on RFLP was 82.2% in healthy dogs and 80.4% in dogs with skin infections (Sareyyupoglu et al. 2014; Findik et al. 2018). Ozturk et al. (2010) identified *Staphylococcus* species in dogs with otitis externa, skin wounds and pyoderma. Nevertheless none of them was *S. pseudintermedius*. They isolated 19.1%, 9.7%, 7.7% *S. intermedius* in dogs with otitis externa, skin wounds, and pyoderma, respectively. However, they used 16S rRNA sequencing for identifying *Staphylococcus* species, which is not recommended by CLSI for discriminating between *S. intermedius, S. pseudintermedius,* and *S. delphini*. Thus, they may not have been able to identify *S. pseudintermedius* within the SIG group.

Methicillin-resistant *S. pseudintermedius* has been reported in companion animals (Ruscher et al. 2010; Weese and van Duijkeren 2010). MRSP isolation rates of 2-7% have also been reported in various European countries, even in healthy dogs (De Lucia et al. 2011; Gomez-Sanz et al. 2011; Nienhoff et al. 2011). In 2019, the proportion of MRSP isolates among clinical samples was 9.3% in one Central European setting (Krapf et al. 2019). In contrast, MRSP isolation rates of 47-66% have been reported in Asian countries (Kawakami et al. 2010; Feng et al. 2012; Bardiau et al. 2013). Turkey has only limited recent data on the frequency of MRSP isolates from canine samples. The frequency of MRSP strains in this study was lower than in Asia but higher than in Europe. The rate of MRSP isolates in the present study was also lower than in a previous study in Turkey (Sareyyupoglu et al. 2014), who reported *S. pseudintermedius* frequencies of 33.3% in shelter dogs with dermatitis. In the present study, 18 isolates were MRSP (29.5%). This decrease in MRSP rates between the two studies can be explained by new regulations that strictly control and restrict antibiotic usage in animals in Turkey.

Multidrug resistance in *S. pseudintermedius* has been reported and is a major concern in Europe. In Germany, Loeffler et al. (2007) reported that 23% of *S. pseudintermedius* isolates were MDR while Findik et al. (2018) reported 13.04% MDR *S. pseudintermedius* strains isolated from healthy dogs in Turkey. In the present study, the MDR *S. pseudintermedius* isolation rate was 40.9% in dogs with skin infections whereas Findik et al. (2018) found no MDR-MRSP isolates in healthy dogs. Menandro et al. (2019) re-

ported that all MRSP isolates (31.6%) in their study were MDR in dogs with pyoderma, conjunctivitis, otitis, or urinary tract or bone infections. In the present study, we detected 22.9% MDR-MRSP. This high level of MDR in MRSP isolates indicates that MRSP isolates usually have an MDR profile. The difference in MDR and MDR-MRSP rates may be due to differences in the animals' health condition. Finally, the identification of isolates only susceptible to amikacin reflects the limited use of amikacin in veterinary practice.

In conclusion, the high frequency of MRSP isolates in dogs in Turkey constitutes a risk for public health. However, restricting the usage of antibiotics decreases antibiotic resistance in *S. pseudintermedius* strains. Comprehensive monitoring is needed to understand the epidemiology of this pathogen and the current status of antibiotic resistance in other Staphylococci.

Conflict of interest

Declaration of interest: N/A

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