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Determination of the diversity and antibiotic resistance profiles of *Staphylococcus* species from dogs with otitis externa and examination of *mecA* gene occurrence

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ABSTRACT: The aim of this study was to determine the distribution of Staphylococci from swab samples of dogs with otitis externa and to determine their antibiotic resistance profiles, particularly methicillin resistance. For this purpose 116 ear swab samples were collected from 100 dogs and examined for the presence of *Staphylococcus* species by conventional culture methods. Antibiotic susceptibility of the isolates was determined by the disk diffusion test and for methicillin resistance, by PCR. Forty Staphylococci were isolated from 37 (31.9%) of the 116 ear swabs. Among the 40 isolates, 30 of them were coagulase-positive *Staphylococcus* species (CPS), while 10 (25%) were coagulase-negative *Staphylococcus* spp. (CNS). *S. pseudintermedius* (*n* = 11), *S. aureus* (*n* = 8), other not determined *Staphylococcus* spp. (*n* = 7), *S. chromogenes* (*n* = 7), *S. schleiferi coagulans* (*n* = 3), *S. hyicus* (*n* = 1), *S. hominis* subsp. *hominis* (*n* = 1), *S. simulans* (*n* = 1), *S. saprophyticus* (*n* = 1) were isolated. Results of the antibiotic susceptibility tests have shown that 60% of the isolates were resistant to sulfamethoxazole/trimethoprim, 32.5% of them were resistant to erythromycin, 25% were resistant to clindamycin, and all isolates (100%) were sensitive to amoxicillin/clavulanic acid and cephazolin. The majority of isolates (97.5%) were sensitive to ciprofloxacin and gentamicin which are frequently used in otitis externa treatment. It was determined that only one (2.5%) (*S. hominis* subsp. *hominis*) of the 40 isolates was resistant to methicillin and carried the *mecA* gene. We found 77% of *Staphylococcus* spp. to be resistant to one or more antimicrobial drugs, and 25% of *Staphylococcus* species were found to be resistant to three or more antimicrobial classes. Thus, multidrug-resistance as detected in our study should always be taken into account and close attention should be given to the antimicrobial therapy protocols of pet animals.

Keywords: dog; otitis externa; *Staphylococcus* spp.; methicillin resistance; *mecA* gene

Staphylococci persist on mucosal surfaces and on skin in a commensal form. However, they also represent the main factor in many animal diseases. Staphylococci are divided into coagulase-positive and coagulase-negative subclasses. The coagulase-positive Staphylococci (CPS) are related with more chronic and sub-acute infections, while the coagulase-negative Staphylococci (CNS) have long been neglected in terms of pathogenic research (Mouney et al. 2013).

S. intermedius has been reported to be the most frequently isolated species among the CPS both in healthy and infected dogs (Sasaki et al. 2007). It has also been reported that *S. aureus* and *S. intermedius* are responsible for many skin problems and otitis externa infections in pet animals (Guardabassi et al. 2004a). There are some reports of isolation of *S. intermedius* both from the ear canal of a dog and from its owner (Tanner et al. 2000; Guardabassi et al. 2004a).

In the last decade it has been suggested that pet animals may constitute a reservoir of antibiotic-resistant bacteria (Guardabassi et al. 2004b; Loeffler et al. 2010a). Isolation of Staphylococci that are resistant to methicillin and other antibiotics has been frequently reported. There are also sporadic reports of the transmission of methicillin-resistant *Staphylococcus aureus* (MRSA) from animals to humans and vice versa (O'Mahony et al. 2005; Duquette and Nuttal 2004; Strommenger et al. 2006; Boost et al. 2008; Loeffler et al. 2010b). Although there are some reports on infections caused by Methicillin-Resistant CNS in cats and

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dogs, the pathogenic potential of these microorganisms in animals has not yet been fully accepted (Van Duijkeren et al. 2004; Epstein et al. 2009). The transfer resistance among dogs, cats, horses and other domestic animals is possible, and these carrier animals are reported to act as reservoirs both for other animals and humans (Weese et al. 2010; Loeffler et al. 2010a).

In this study, the purpose was to determine the distribution of *Staphylococcus* species isolated from dogs with otitis externa, to establish their antibiotic resistance profiles, to determine the *Staphylococcus* species that are resistant to methicillin and to examine the presence of the *mecA* gene with PCR.

MATERIAL AND METHODS

In this study, 116 ear swab samples collected from 100 dogs with ear canal complaints were examined. The samples were collected from dogs brought to private veterinary clinics located in different districts of Istanbul or to clinics of Istanbul University, Faculty of Veterinary Medicine. In addition, some of the dogs were living in dog shelters.

The swab samples were inoculated into Nutrient Broth (Oxoid Ltd, Hampshire, England) supplemented with 1% horse serum, and incubated at 37 °C under aerobic conditions. After a 24–48 h incubation period, passages were performed onto mannitol-salt agar (MSA, Oxoid), and onto blood agar base (Oxoid) containing 5% sheep blood. Pure Gram-positive cultures were examined for *Staphylococcus* spp. identification, by performing coagulase, catalase, oxidase, DNase, haemolysis, pigment production, urease activity, mannitol fermentation, esculin hydrolysis, novobiocin and polymyxin B antibiotic susceptibility tests (Holt et al. 1994). The results were confirmed by API ID32 STAPH (Bio-Merieux Co. Ltd., France). In order to discriminate 18 isolates which were identified as *Staphylococcus intermedius* group (SIG; *S. intermedius*, *S. delphini* or *S. pseudintermedius*) phenotypically, a multiplex-PCR was performed. Bacterial DNA was extracted using the QIAamp DNA extraction kit (Qiagen, Cat. No.69506). The PCR reaction mixture consisted of 2 µl of DNA extract in a total volume of 50 µl and was performed according to Sasaki et al. (2010). DNA fragments were analysed by electrophoresis in 1 × Tris-acetate-EDTA on a 1% agarose gel stained with ethidium bromide. *Staphylococcus intermedius*: (CCM5729), *Staphylococcus pseudintermedius*: (NVAV-6045), *S. delphini* group B, (H-2C) and *S. delphini* group A; (F-13b) were used as positive controls.

The antibiotic resistance of the isolates was examined according to the standards of the Clinical and Laboratory Standards Institute (CLSI) using the following antibiotic disks: amikacin (30 µg), amoxicillin/clavulanic acid (20/10 µg), ampicillin (10 µg), cefazolin (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), enrofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), lincomycin (2 µg), neomycin (30 µg), penicillin G (10 µg), rifampicin (5 µg), sulfamethoxazole/trimethoprim (1.25/23.75 µg), tetracycline (30 µg), tobramycin (10 µg), vancomycin (30 µg), oxacillin (1 µg), (CLSI 2010). Methicillin resistance was confirmed by determining the presence of the *mecA* gene in a PCR assay carried out according to Jonas et al. (2002).

RESULTS

A total of 116 swab samples were examined from 100 dogs with otitis externa. In 37 samples bacterial growth was observed; 40 *Staphylococcus* species were isolated from those 37 ear swabs. It was determined that 30 of these isolates (75%) were CPS, while 10 (25%) were CNS (Table 1). Eight *S. aureus* (20%), seven *S. chromogenes* (17.5%), three *S. schleiferi coagulans* (7.5%), one *S. hyicus* (2.5%), one *S. hominis* subsp. *hominis* (2.5%), one *S. simulans* (2.5%), one *S. saprophyticus* (2.5%) and eighteen SIG (42.5%) isolates were recovered. Eleven out of the 18 SIG isolates were determined to be *S. pseudintermedius* after PCR analysis. In the remaining seven isolates which were identified as *S. intermedius* by biochemical tests and API no bands were observed; these were idenfied as *Staphylococcu*s spp. (Figure 1).

It was determined that 60% of the isolates were resistant to sulfamethoxazole/trimethoprim and all isolates (100%) were sensitive to amoxicillin/clavulonic acid. The majority of isolates (97.5%) were sensitive to ciprofloxacin and gentamicin which are frequently used in otitis externa treatment. One of (*S. hominis* subsp. *hominis*) the isolates was determined to be methicillin-resistant by both the disk diffusion method and PCR (Figure 2). This isolate

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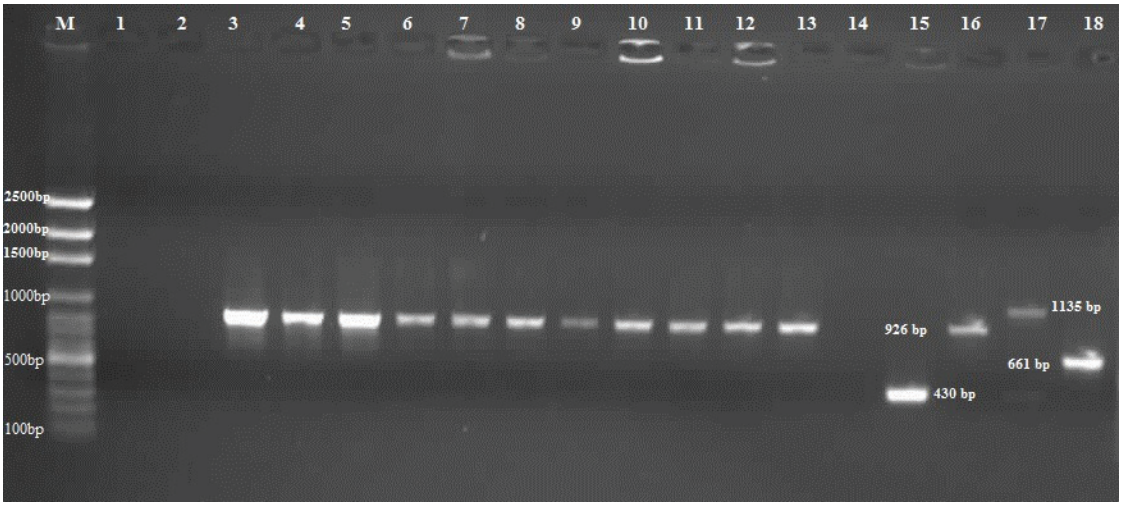

Figure 1. Multiplex-PCR products on a 1% agarose gel. M = DNA molecular weight marker (100 bp); line 1, 2 = *Staphylococcus* spp.; lines 3–13 = *Staphylococcus pseudintermedius* 926 bp band; line 14 = negative control; line 15 = *Staphylococcus intermedius* (CCM5729), positive control, 430 bp; line 16 = *Staphylococcus pseudintermedius* (NVAV-6045), positive control, 926 bp; line 17 = *S. delphini* (H-2C), group B, positive control, 1135 bp; line 18 = *S. delphini* (F-13b), group A, positive control, 661 bp

was also multidrug resistant; it was resistant to ampicillin, ciprofloxacin, enrofloxacin, kanamycin, gentamicin, tetracycline, tobramycin, erythromycin, sulfamethoxazole/trimethoprim (Table 1). Other than this, an additional 10 Staphylococci isolates were determined to be multidrug resistant (resistant to three or more antibiotic groups). Additionally, among eight isolates the same resistance pattern - resistance to clindamycin, erythromycin and sulfamethoxazole/trimethoprim, was observed (Table 1). Results of antibiotic susceptibility tests are shown in Table 2.

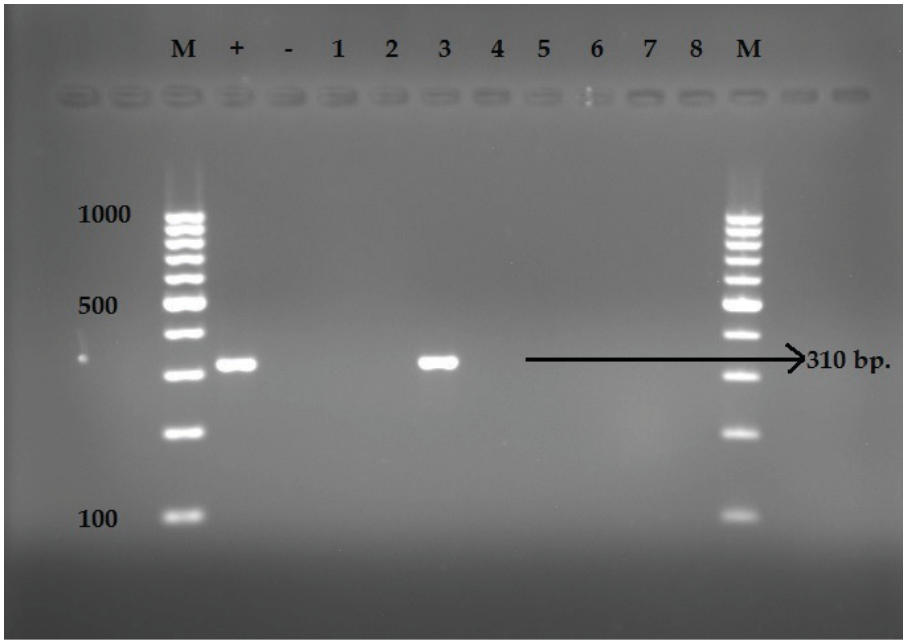

Figure 2. The 310 bp amplicon of the *mecA* gene; 2% agarose gel. PCR analysis for the *mecA* gene in staphylococci isolated from dogs with external otitis. M = DNA molecular weight marker (100 bp); + = *S. aureus* (*mecA*, positive, 310 bp); – = negative control; line 3 = *mecA* positive isolate (*Staphylococcus hominis* subsp. *hominis*, 310 bp); lines 1, 2, 4, 5, 6, 7, 8 = *mecA*, negative isolates

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Table 1. Resistance pattern of strains of staphylococci species isolated from otitis externa in dogs

	Resistant isolates	Antimicrobial resistance pattern (*n* isolates)
CPS organism (*n*)		
S. aureus (8)	5/8, 62.5%	CD + E + K + N + P + SXT + T + TB (1), CD + E + K + SXT + P (1), SAM + AM (1), AM + G (1), SXT (1)
S. pseudintermedius (11)	7/11, 63.6%	AM + CD + E + K + L + SXT + T (1), CD + E + K + N + SXT (1), CD + E + K + SXT (1), SXT + T (2), AM + T (1), AM (1), SXT (2)
Staphylococcus spp. *(SIG)* (7)	7/7, 100%	CD + E + L + SXT (1), CD + E + SXT (1), E + SXT (1), T + RD (1), AM (1), SXT (2)
S. schleiferi coagulans (3)	2/3, 66. 7%	SXT + T (1), SXT (1)
S. hyicus (1)	1/1, 100%	SXT (1)
CNS organism (*n*)		
S. chromogenes (7)	4/7, 57.1%	AM + CD + E + K + N + SXT + T + TB (1), CD + E + L + N + P + RD + SXT + V (1), E + N (1), CD (1)
S. hominis subsp. *hominis* (1)	1/1, 100%	A + CRO + CF + ENR + E + G + K + SXT + T + TB + OX (1)
S. simulans (1)	1/1, 100%	SXT (1)
S. saprophyticus (1)	1/1, 100%	E + SXT (1)

CRO = ceftriaxone, CF = ciprofloxacin, CD = clindamycin, ENR = enrofloxacin, E = erythromycin, G = gentamicin, K = kanamycin, L = lincomycin, N = neomiycin, OX = oxacillin, P = penicillin G, RD = rifampicin, SXT = sulfamethoxazole/trimethoprim, T = tetracycline, TB = tobramycin, V = vancomycin

DISCUSSION

Otitis externa is a frequent problem in dogs and there have been many studies conducted on the aetiology of this disease. *S. aureus* and *S. intermedius* group were the most common strains identified in those studies (Keskin et al. 1999; Sarierler and Kirkan 2004; Strommenger et al. 2006; Boost et al. 2008; Ozturk et al. 2010).

Table 2. Results of antimicrobial susceptibility tests

Antimicrobial agents tested	Resistant isolates (*n*)	Rate (%)
Sulphamethoxazole/Trimethoprim	24	60
Erythromycin	13	32.5
Clindamycin	10	25
Ampicillin	8	20
Kanamycin	8	20
Tetracycline	8	20
Lincomycin	4	10
Neomycin	4	10
Tobramycin	3	7.5
Penicillin G	2	5
Gentamicin	1	2.5
Amicacin	1	2.5
Ceftriaxone	1	2.5
Ciprofloxacin	1	2.5
Enrofloxacin	1	2.5
Vancomycin	1	2.5
Oxacillin	**1**	**2.5**
Amoxicillin/clavulanic acid	0	0
Cephazolin	0	0
Rifampicin	0	0

Some researchers have reported that the isolation rate of members of the *Staphylococcus intermedius* group is higher than other staphylococci, while it has also been shown that among SIG, *S. pseudointermedius* isolation is higher than other SIG members (Futagawa-Saito et al. 2006; Jones et al. 2007; Sasaki et al. 2007; Vanni et al. 2009). Similarly, in our study *S. pseudintermedius* was the most common staphylococcus detected from ear swabs.

Studies on methicillin-resistant staphylococci are focused mainly on farm animals, in Turkey. There have also been a few studies in dogs (Findik et al. 2009; Bagcigil et al. 2012). Findik et al. (2009) reported the isolation of *S. aureus* from 80 (20.5%) swab samples from 390 dogs and detected the *mecA* gene only in three (3.8%) isolates. Bagcigil et al. (2012) isolated methicillin-resistant coagulase negative staphylococci (MRCNS) from five dogs (23.8%) out of 21 dogs. In the current study, only one methicillin-resistant *S hominis* subsp. *hominis* isolate was detected both phenotypically and genotypically. Although the rate of *S. pseudintermedius* and *S. aureus* isolation was relatively high, the absence of the *mecA* gene was reassuring.

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Ozturk et al. (2010) reported that only five (9.3%) of the 54 CPS isolated from 96 dogs with otitis externa, pyoderma, and skin wounds were methicillin-resistant phenotypically, and they did not carry the *mecA* gene. We did not detect any methicillin-resistant CPS isolates. Some studies performed in dogs have also reported a similarly low prevalence (Vanderhaeghen et al. 2012; Wedley 2012).

It has been reported that resistance rates to penicillin G, erythromycin, tetracycline and fusidic acid among *S. aureus* isolates is high, and it was proposed that the acquisition of resistance to these antibiotics was due to their higher usage by veterinarians (Boost et al. 2008). *Staphylococcus* species isolated from dogs with pyoderma were found to be resistant to streptomycin, kanamycin, neomycin and erythromycin (28%), to clindamycin (22%) and to gentamicin and enrofloxacin (2%; Boost et al. 2008).

Keskin et al. (1999) reported that 82.5% of the bacteria isolated from the dogs with otitis externa were resistant to enrofloxacin, 65.5% to cephalosporins, 44.4% to gentamicin and tetracycline, 34.9% to spiramycin, 26.9 to ampicillin, while 20.6% were resistant to lincomycin. Sarierler and Kirkan (2004) reported that bacteria isolated from dogs with otitis externa were resistant to oxytetracycline (100%), ciprofloxacin (100%), kanamycin (87.5%), penicillin G (72.5%), erythromycin (57.5%), gentamicin (55%), ampicillin (50%) and cefoperazone (50.0%). In our study, 2.5% of the isolates were resistant to gentamicin and ciprofloxacin which are frequently used in the treatment of dogs with otitis externa. The susceptibility rates of the isolates to ciprofloxacin were close to the rates obtained in the study by Sarierler and Kirkan (2004). In a study conducted in our country on dogs with otitis externa it was reported that *Staphylococcus* species were highly resistant (63.1%) to ampicillin (Keskin et al. 1999). In our study, we determined that the resistance rate to ampicillin was 20%. The difference between these results is striking. In our study, the highest resistance rates we detected were to sulfamethoxazole/trimethoprim (60%), erythromycin (32.5%), clindamycin (25%), and penicillin G (5%), respectively. We consider that in recent years the use of penicillin G in cats and dogs has decreased, while that of sulfamethoxazole/trimethoprim and erythromycin has increased, and therefore resistance has developed over time. The resistance rates to clindamycin and gentamicin detected in our study are in accordance with those detected by Ganiere et al. (2005).

Van Duijkeren et al. (2004) reported that all 10 *mecA*-positive Staphylococci strains bar one were multidrug-resistant and warned that if they were resistant to all antibiotic types used in dogs, the infections would be difficult to treat. We determined in our study that the isolate which was detected as *mecA*-positive was multi-resistant. The importance of multidrug-resistance among bacteria is increasing in both human and veterinary medicine. Ganiere et al. (2005) found 41 of the 50 *S. intermedius* strains (82%) to be resistant to one or more antimicrobial drugs and 21 strains (42%) to be resistant to three or more antimicrobial classes. We found 31 of the 40 *Staphylococcus* spp. (77.7%) to be resistant to one or more antimicrobial drugs and 10 *Staphylococcus* species (25%) were observed to be resistant to three or more antimicrobial classes. This multidrug-resistance should not be ignored and the necessary importance and attention should be given to the antimicrobial therapy protocols of pet animals.

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