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RESEARCH ARTICLE

Methicillin Resistance Among Coagulase-positive Staphylococci Isolated from Dogs with Otitis Externa, Skin Wounds and Pyoderma [1]

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Summary

In this study, 54 coagulase-positive staphylococci (33 *Staphylococcus aureus* and 21 *Staphylococcus intermedius*) isolated from 96 dogs with otitis externa, skin wounds and pyoderma were investigated for the methicillin (oxacillin) resistance. *S. aureus* was detected as the predominant coagulase-positive staphylococci in dogs affected by otitis externa and skin infections. Among coagulase-positive staphylococci, four *S. aureus* and one *S. intermedius* isolates, which did not contain *mec*A gene were found phenotypically resistant to methicillin, but were susceptible to amoxicillin/clavulanic acid by the disk diffusion test. Five phenotypic methicillin-resistant coagulase-positive staphylococci were isolated from two (2.2%) dogs with otitis externa, two (15.4%) dogs with pyoderma and one (3.2%) dog with skin wound. The results of this study shows that methicillin-susceptible or -resistant *S. aureus* and *S. intermedius* are the predominate organisms in dogs with otitis externa and skin infections, and the rapid and correct diagnosis of methicillin-resistance is of great importance for the treatment of dogs.

Keywords: *Dog, Otitis externa, Skin wounds, Pyoderma, Coagulase-positive staphylococci, Methicillin resistance*

Otitis Eksterna, Deri Yarası ve Pyodermalı Köpeklerden İzole Edilen Koagülaz Pozitif Stafilokokların Metisilin Dirençliliği

Özet

Bu çalışmada, otitis eksterna, deri yarası ve pyodermalı 96 köpekten elde edilen 54 koagülaz-pozitif stafilokok (33 *Staphylococcus aureus* ve 21*Staphylococcus intermedius*) izolatı metisilin (oksasilin) direnci yönünden incelendi. Otitis eksterna ve deri enfeksiyonu tespit edilen köpeklerden en fazla izole edilen koagülaz-pozitif stafilokokun, *S. aureus* olduğu belirlendi. Koagülaz-pozitif stafilokoklar arasında disk diffüzyon testi ile metisiline fenotipik olarak dirençli bulunan dört *S. aureus* ve bir *S. intermedius* izolatının *mec*A geni taşımadıkları ve amoksisilin/klavulanik aside duyarlı oldukları saptandı. Fenotipik metisilin direnci belirlenen beş koagülaz-pozitif stafilokok izolatının ikisi (%2.2) otitis eksterna, ikisi (%15.4) pyoderma ve biri de (%3.2) deri yarasına sahip köpeklerden elde edildi. Çalışmadan elde edilen sonuçlar, otitis eksterna ve deri enfeksiyonlarına sahip köpeklerde en yaygın izole edilen etkenlerin metisiline duyarlı veya dirençli *S. aureus* ile *S. intermedius* olduğunu ve metisilin direncinin hızlı ve doğru bir şekilde teşhis edilmesinin, bu köpeklerin tedavisi için büyük öneme sahip olduğunu göstermektedir.

Anahtar sözcükler: *Köpek, Otitis eksterna, Deri yarası, Pyoderma, Koagülaz-pozitif stafilokok, Metisilin dirençliliği*

INTRODUCTION

Staphylococcal species occur as commensals on mucous membranes and skin of animals and man [1-4]. Coagulase-positive staphylococci (CoPS, *Staphylococcus aureus* and *Staphylococcus intermedius*) are responsible for the majority of domestic animal infections [3-6]. *S. intermedius* is commonly isolated from dogs with

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pyoderma, otitis externa and suppurative conditions including mastitis, endometritis, cystitis, osteomyelitis and wound infections [1,3,6-8]. Occasionally, similar suppurative conditions are caused by *S. aureus* [3,4,9-12].

Staphylococci that are resistant to semi-synthetic penicillins such as methicillin, oxacillin or cloxacillin are considered to have methicillin resistance. Methicillin resistance is an alarming condition for treatment because it implies resistance not only to all beta-lactam antibiotics including cephalosporins, but also to a wide range of antibiotics [3,5,13,14]. Methicillin resistance in staphylococci is mediated by *mecA* gene, which encodes a penicillin-binding protein 2a (PBP-2a) with low affinities for β-lactam antibiotics [13,14]. In routine applications, detection of methicillin resistance among staphylococci is generally based on phenotypic assays [3,14-16]. However, genetic confirmation for the presence of *mecA* gene is essential for detection of methicillin resistance in all staphylococci [13-15,17]. The *fem*A, that acts as a regulator gene for the expression of the methicillin resistance has been reported as a valuable tool for species identification of *S. aureus* [14,17-20].

Methicillin (oxacillin)-resistant *Staphylococcus* species, especially methicillin-resistant *S. aureus* (MRSA) isolates, have been generally isolated from human beings [5,12,14,21]. In recent years, increasing numbers of reports have documented the occurrence of MRSA in various animal species [8,10,22-26]. It has been suggested that MRSA can cause infections in dogs, and dogs can also act as reservoirs of MRSA [8,10,22,23,25,27-29].

In dogs, in contrast to human, the number of studies carried out on clinical infections caused by methicillin-resistant staphylococci (MRS) is limited [8-11,26,27,30-34]. Except a few study [30,31,34], any of these studies are not focused on selected cases of the canine pyoderma and otitis externa which are major infections of CoPS in dog. The aim of the present study was to determinate the methicillin resistance profile of CoPS isolated from dogs with otitis externa, skin wounds and pyoderma.

MATERIAL and METHODS

Animals

A total of 96 dogs with 52 otitis externa, 31 skin wounds and 13 pyoderma were sampled between January 2007 and January 2009. These dogs were brought to the Small Animal Clinic (Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Burdur, Turkey) by owners (n=39) or local animal shelter staffs (n=57) for diagnosis and treatment. All dogs were examined clinically and were also subjected to otoscopic examination for the diagnosis of otitis externa. One hundred thirty three swap samples for culture were taken from either the ear canals of dogs with otitis externa (n=89) or the skin lesions of dogs with wounds (n=31) and pyoderma (n=13). Thirty-seven of 52 dogs had bilateral otitis externa. None of the dogs was under treatment for a certain infection. The dogs ranged in age from 1 to 12 years. Among the sampled 96 dogs, 67 were of mix-breed and the remaining 29 were from different breeds (8 Terriers, 7 Anatolian Shepherd Dogs, 6 Pointers, 2 Doberman Pinchers, 2 Golden Retrievers and and one of each breed of French Bulldog, Siberian Husky, Irish Setter and American Cocker).

Sampling and Isolation of Staphylococci

All the swabs were streaked on 5% sheep blood agar (Oxoid Ltd, Hampshire, England), MacConkey agar (Oxoid), and Sabouraud's dextrose agar (Oxoid) plates. Blood agar and MacConkey agar plates were incubated at 37°C for 24 to 48 h under aerobic conditions. Sabouraud's dextrose agar (with chloramphenicol) plates that were plated for fungus and yeast isolation were incubated at 25°C for 7 days. After presumptive identification based on colony morphology and microscopic morphology, biochemical and growth characteristics of the isolates were determined [4,35]. Staphylococci were identified according to the conventional methods, including Gram staining, colony morphology, haemolysis, and tests for catalase, clumping factor, tube coagulase, DNAse, acetoin and anaerobic fermentation of mannitol. The discrimination between *S. aureus* and *S. intermedius* was achieved using the Voges-Proskauer reaction (acetoin production) [4,35].

In PCR and antimicrobial susceptibility testing, *mecA*-positive *S. aureus* 27R (Hacettepe University, Faculty of Medicine, Department of Microbiology and Clinical Microbiology, Ankara, Turkey) and *mecA*-negative *S. aureus* ATCC 25923 (Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Microbiology, Burdur, Turkey) were used as control strains. These strains were stored at -20°C in trypticase soy broth (TSB, Oxoid) containing 10% glycerol. Prior to testing, all isolates were serially cultured twice on blood agar plates containing 5% sheep blood and incubated for 24 h at 37°C under aerobic conditions.

Antimicrobial Susceptibility Testing

Phenotypic methicillin (oxacillin) resistance was determined by a disk diffusion method on Mueller-Hinton agar (Oxoid) according to the Clinical Laboratory Standards Institute (formerly National Committee of Clinical Laboratory Standards, NCCLS) [16] standards. Ten colonies from blood agar base containing 5% sheep blood incubated at 37°C for 18 h were suspended in sterile saline to a density approximately equal to McFarland

Opacity Standard No. 0.5. The bacterial suspension was inoculated onto Mueller-Hinton agar containing 2% NaCl with the swab to cover the whole surface of the agar. The oxacillin (1 µg, Oxoid) disks were dispensed on the surface of the media and were incubated aerobically at 35°C for 24 h. The results were recorded as susceptible (≥13 mm), intermediate susceptible (11-12 mm) or resistant (≤10 mm) by measurement of the inhibition zone diameter according to the interpretive standards of NCCLS [16]. The reference strains used for antibiotic susceptibility assays were *S. aureus* 27R and *S. aureus* ATCC 25923. Additionally, the susceptibility of *S. aureus* isolates to amoxicillin/clavulanic acid (20/10 µg, Oxoid) was tested to identify the β-lactamase-producing isolates [16].

DNA Extraction from Culture Samples

Single colonies of *S. aureus* and *S. intermedius* isolates were inoculated into Brain Hearth Infusion (BHI) and incubated at 37°C for 20 h. TSB cultures (approximately 10^9 bacteria per ml) were pelleted by centrifugation (12.000 rpm for 10 min). Bacterial pellet was resuspended in 200 µl of sterile distilled water and mixed by vortex shortly. The suspension was incubated at 100°C for 10 min and cooled. After gently mixing by vortex, the suspension was centrifuged (12.000 rpm for 10 min). Then, the supernatant was collected and the total solution was transferred to a micro test tube. Isolated DNA samples were kept at -20°C until use [24].

PCR

S. aureus and *S. intermedius* isolates were analyzed by PCR for the presence of the gene for methicillin resistance (*mecA*) and a gene (*femA*) used for species identification of *S. aureus.* Primers for *mecA* and *femA* were chosen from published sequences [20,36] *(Table 1).* Deoxyribonucleotide triphosphate (dNTP), Taq DNA polymerase enzyme and buffers used in PCR mixture were supplied by the manufacturer (Applied Biosystem, Roche, USA). The assay was performed in a final volume of 25 µl reaction mixture consisted of 5 µl template DNA, 12.5 µl 2X PCR mastermix, 1 µl primer F (100 pmol), 1 µl primer R (100 pmol) and 5.5 µl ddH_2O. The amplification was carried out in a thermal cycler (CLP, ATC401, USA) under the following conditions:

mecA: DNA denaturation step of 5 min at 95°C; 30 cycles with a 2 min denaturation step at 95°C, a 30 s annealing step at 54°C, and a 30 s extension at 72°C and a final 5 min extension step at 72°C.

femA: DNA denaturation step of 5 min at 94°C; 35 cycles with a 45 s denaturation step at 94°C, a 45 s annealing step at 54°C, and a 45 s extension at 72°C and a final 5 min extension step at 72°C.

After amplification, PCR products (10 µl) were electrophoresed in 1.5% agarose gel at 100 V for 45 min, stained with ethidium bromide (0.5 µg/ml) and photographed under UV light (Edas 290, Eastman Kodak Company, Rochester, NY, USA). The PCR analyses of all isolates were performed in duplicate. The control organisms (*S. aureus* 27R and *S. aureus* ATCC 25923) were also included in PCR assays.

Table 1. *Primer sequences used in PCR and the expected sizes of the products*

Tablo 1. *PCR'da kullanılan primer dizilimler ve beklenen büyüklükleri*

Target Genes	Primer Sequence (5' - 3')	Size (bp)	Ref.
*mec*A	Forward-CCTAGTAAAGCTCCGGAA Reverse-CTAGTCCATTCGGTCCA	314	36
*fem*A	Forward-AAAAAAGCACATAACAAGCG Reverse-GATAAAGAAGAAACCAGCAG	132	20

RESULTS

Isolation of Staphylococci

Staphylococci were isolated from 44 of swap samples of dogs with otitis externa, 14 with skin wounds and 7 with pyoderma, yielding a total of 54 CoPS and 11 coagulase-negative staphylococci (CoNS) isolates.

Table 2. *The number and rate of microorganisms isolated from dogs with otitis externa, skin wounds and pyoderma*

Tablo 2. *Otitis eksterna, deri yarası ve pyodermalı köpeklerden izole edilen mikroorganizmaların sayısı ve oranı*

Genus/Species	No of Isolates	%
Otitis externa (n=89)		
Staphylococcus aureus	21	23.6
Staphylococcus intermedius	17	19.1
Candida spp.	11	12.4
Coagulase-negative staphylococci	6	6.7
Streptococcus spp.	2	2.3
Escherichia coli	1	1.1
Malassezia pachydermatis	2	2.3
Pythium insidiosum	1	1.1
No growth	28	31.4
Skin wounds (n=31)		
Staphylococcus aureus	7	22.5
Staphylococcus intermedius	3	9.7
Coagulase-negative staphylococci	4	12.9
Candida spp.	2	6.5
Escherichia coli	2	6.5
No growth	13	41.9
Pyoderma (n=13)		
Staphylococcus aureus	5	38.5
Staphylococcus intermedius	1	7.7
β-haemolytic streptococci	4	30.8
Coagulase-negative staphylococci	1	7.7
No growth	2	15.3

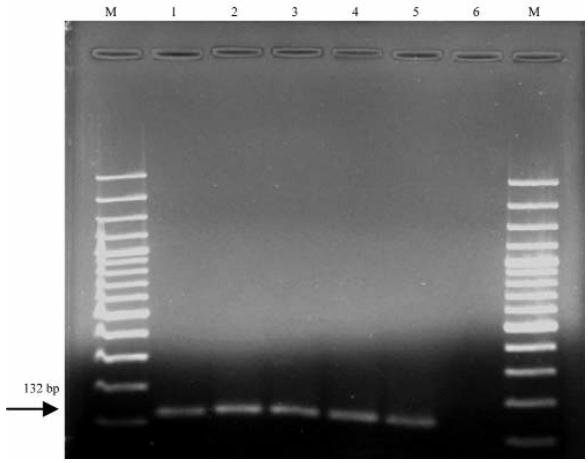

Fig 1. PCR results for *fem*A gene of the phenotypically methicillin-resistant CoPS isolated from dogs. Lane M, DNA molecular weight marker (100 bp); lane 1, 27R *S. aureus* (*mecA*-positive); lane 2-5, *S. aureus* isolates; lane 6, *S. intermedius* isolate

Şekil 1. Köpeklerden izole edilen metisiline fenotipik dirençli CoPS'ların *fem*A geni yönünden PCR sonuçları. Sütun M: DNA moleküler ağırlık işaretleyicisi (100 bp); sütun 1, 27R *S. aureus* (*mecA*-pozitif); sütun 2-5, *S. aureus* izolatları; sütun 6, *S. intermedius* izolatı

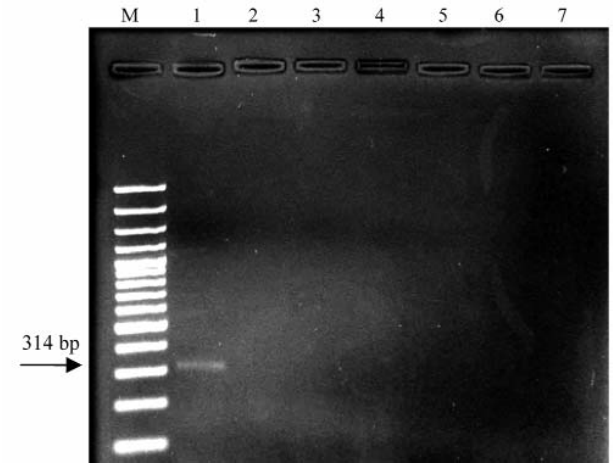

Fig 2. PCR results for *mec*A gene of the phenotypically methicillin-resistant CoPS isolated from dogs. Lane M, DNA molecular weight marker (100 bp); lane 1, 27R *S. aureus* (*mecA*-positive); lane 2, 25923 *S. aureus* (*mec*A-negative); lane 3-6, *S. aureus* isolates; lane 7, *S. intermedius* isolate

Şekil 2. Köpeklerden izole edilen metisiline fenotipik dirençli CoPS'ların *mec*A geni yönünden PCR sonuçları. Sütun M: DNA moleküler ağırlık işaretleyicisi (100 bp); sütun 1, 27R *S. aureus* (*mecA*-pozitif); sütun 2, 25923 *S. aureus* (*mecA*-negatif); sütun 3-6, *S. aureus* izolatları; sütun 7, *S. intermedius* izolatı

Fourteen of 37 dogs with bilateral otitis externa were infected with the same microorganisms. Coagulase-positive *S. aureus* and *S. intermedius* were detected as the predominant organisms in dogs. The isolation of both CoPS and CoNS were more frequent from otitis externa samples than skin lesions of dogs with pyoderma and wounds. The isolation rates of microorganisms from dogs with clinical infections were reported in *Table 2.*

Phenotypic and Genotypic Resistance to Oxacillin

Most of isolated staphylococci were phenotypically oxacillin-susceptible, coagulase-positive *S. aureus* (n=29) and *S. intermedius* (n=21) isolates. Of the 33 *S. aureus* isolates, 4 (12.1%) were resistant to oxacillin by the disk diffusion test. Two of these 4 isolates were recovered from otic samples. The remaining two isolates were from skin lesions. Only one (4.8%) of 21 *S. intermedius* isolates was also resistant to oxacillin.

The PCR correctly determined the presence or absence of the genes of interest in the reference strains. The *femA* gene was detected in all of 33 *S. aureus* isolates by PCR, but was undetectable in all of *S. intermedius* isolates tested *(Fig. 1).* All of CoPS isolates were negative for *mecA* gene *(Fig. 2).* Five staphylococci which were resistant to oxacillin were susceptible to amoxicillin/clavulanic acid after repeated disk diffusion testing. Details of the isolates are shown in *Table 3.*

Table 3. *Phenotypic and genotypic methicillin resistance of 33 S. aureus and 21 S. intermedius isolates from dogs with otitis externa, skin wounds and pyoderma*

Tablo 3. *Otitis eksterna, deri yarası ve pyodermalı köpeklerden elde edilen 33 S. aureus ve 21 S. intermedius izolatının fenotipik ve genotipik metisilin dirençliliği*

Dog Source	No of Samples	Isolates					
		S. aureus			*S. intermedius*		
		No of Isolates	Resistant to Oxacillin	*mecA*$^+$	No of Isolates	Resistant to Oxacillin	*mecA*$^+$
Otitis externa	89	21	2	0	17	0	0
Skin wounds	31	7	1	0	3	0	0
Pyoderma	13	5	1	0	1	1	0
Total	133	33	4	0	21	1	0

DISCUSSION

After several reports have presented information suggesting that animals may serve as reservoirs for MRSA infection of humans [22,23,25], the occurrence of

MRSA in dogs have documented more frequent in recent years [5,11,12,27-29]. However, very little is known about the clinical significance of MRS for dogs [30,31,37-39]. In the current study, we investigated the presence of methicillin-resistant CoPS in selected clinical infections of dogs such as otitis externa, skin wounds and pyoderma.

Most of the staphylococci obtained from diseased dogs in this study were coagulase-positive species (*S. aureus* and *S. intermedius*), in agreement with previously reports [6,10,26,30,31,34]. In dogs, although *S. intermedius* is the most prevalent pathogenic *Staphylococcus* spp., several researchers [1,9,21,32] have been reported that the infections with *S. aureus* can occur and may be increasing in prevalence. In the present study, *S. aureus* detected as the predominant CoPS from dogs with otitis externa and skin lesions. This result may be explained with reports of Middleton et al.[32] and Rich et al.[33], who stated that *S. aureus* infections were most prevalent among canine patients.

The most common bacterial agents isolated from ear canals [7,40] and skin lesions [1,2,11,41] of dogs are CoPS. However other bacterial agents have also been isolated including CoNS, beta-haemolytic streptococci, *Corynebacterium* spp, *Proteus* spp. and *E. coli* [7,40,41]. In the present study, the other bacterial organisms such as CoNS, *Streptococcus* spp. and *E. coli* were also involved in clinical infections of dogs. CoNS isolates were less common causes of infections, this result probably reflects that CoNS are opportunistic pathogens of skin and mucosae in dogs as previously reported [26,30,34,42]. Thus, it has been stated that CoNS were isolated both from healthy dogs [26,38,42] and dogs with otitis or pyoderma [26,38].

In this study, we used the PCR technique to detect the presence of *mecA* gene described as a molecular marker of methicillin resistance in *S. aureus* and *S. intermedius* isolates from dogs with clinical infections. But the *mecA* gene was not detected in all CoPS, including the methicillin-resistant isolates *in vitro*. Phenotypic methicillin resistance may appear in staphylococci which lack the *mecA* gene, because they produce large amounts of β-lactamase which results in decreased susceptibility to methicillin [14,21,36]. Addition of β-lactamase inhibitors such as sulbactam or clavulanic acid may help overcome these types of resistance [13,15,21]. Therefore, we thought the antibiotic susceptibility patterns can be influenced by the overproduction of β-lactamase, because all oxacillin resistant isolates were susceptible to amoxicillin/clavulanic acid. The *femA* gene has been reported as valuable tools for species identification of *S. aureus* [14,17-20]. In our study, besides the biochemical characteristics, the *femA* gene was used for genotypic confirmation of *S. aureus* isolates, and this target gene was also determined by PCR in all *S. aureus* isolates. Therefore, we considered that the *fem*A gene may appear to be a unique feature for differential diagnosis between *S. aureus* and other staphylococci as previously reported [14,17-20].

Most of MRSA [8-12,26-30,33,41] and methicillin-resistant *S. intermedius* [30,31,39,41] isolates in dogs have been associated with clinical samples from various infections. In this study, 5 phenotypically methicillin-resistant CoPS (4 *S. aureus* and 1 *S. intermedius*) isolates, which did not contain *mecA* were isolated from two (2.2%) dogs with otitis externa, two (15.4%) dogs with pyoderma and one (3.2%) dog with skin wound. This finding supports that phenotypically oxacillin resistant staphylococci can be caused clinical infections in dogs as previously reported [8,9,32,39]. Also, our findings are supported by those of Gortel et al. [30], Van Duijkeren et al. [10], and Baptiste et al. [27], who reported that most of MRS isolated from dogs with clinical infections is identified as *S. aureus.*

In conclusion, we detected that methicillin-susceptible and -resistant *S. aureus* and *S. intermedius* are the predominate organisms in dogs with otitis externa, skin wounds and pyoderma. Because of the significant increases in the prevalence of MRS, the rapid and correct diagnosis of MRS infections is of great importance for the treatment of dogs. The PCR technique has many useful implementations for rapid and specific detection of the staphylococcal isolates and resistance genes. In addition, surveillance and infection control programs for MRS should be practiced in veterinary medicine.

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