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

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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 mecA 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

INTRODUCTION

Staphylococcal species occur as commensals on mucous membranes and skin of animals and man. Coagulase-positive staphylococci (CoPS, Staphylococcus aureus and Staphylococcus intermedius) are responsible for the majority of domestic animal infections. S. intermedius is commonly isolated from dogs with...
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pyoderma, otitis externa and suppurrative conditions including mastitis, endometritis, cystitis, osteomyelitis and wound infections. Occasionally, similar suppurrative conditions are caused by *S. aureus*. 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. Methicillin resistance in staphylococci is mediated by *mecA* gene, which encodes a penicilllin-binding protein 2a (PBP-2a) with low affinities for beta-lactam antibiotics. In routine applications, detection of methicillin resistance among staphylococci is generally based on phenotypic assays. However, genetic confirmation for the presence of *mecA* gene is essential for detection of methicillin resistance in all staphylococci. The *femA*, 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*.

Methicillin (oxacillin)-resistant *Staphylococcus* species, especially methicillin-resistant *S. aureus* (MRSA) isolates, have been generally isolated from human beings. In recent years, increasing numbers of reports have documented the occurrence of MRSA in various animal species. It has been suggested that MRSA can cause infections in dogs, and dogs can also act as reservoirs of MRSA.

In dogs, in contrast to human, the number of studies carried out on clinical infections caused by methicillin-resistant staphylococci (MRS) is limited. Except a few studies, none 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 determine 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 mixed-breed and the remaining 29 were from different breeds (8 Terriers, 7 Anatolian Shepherd Dogs, 6 Pointers, 2 Doberman Pinchers, 2 Golden Retrievers 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. 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).

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) 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
Opacit 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 10⁸ 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 suspension 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 ddH₂O. 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.

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

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

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

<table>
<thead>
<tr>
<th>Target Genes</th>
<th>Primer Sequence (5’ - 3’)</th>
<th>Size (bp)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>mecA</td>
<td>Forward-CTAGTAAAGCTCGGGA</td>
<td>314</td>
<td>36</td>
</tr>
<tr>
<td>femA</td>
<td>Forward-AAAAAGCGACATAACAAACGC</td>
<td>132</td>
<td>20</td>
</tr>
</tbody>
</table>

**References**

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) 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**

<table>
<thead>
<tr>
<th>Dog Source</th>
<th>No of Samples</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No of Isolates</td>
</tr>
<tr>
<td>Otitis externa</td>
<td>89</td>
<td>21</td>
</tr>
<tr>
<td>Skin wounds</td>
<td>31</td>
<td>7</td>
</tr>
<tr>
<td>Pyoderma</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>133</td>
<td>33</td>
</tr>
</tbody>
</table>

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.

**DISCUSSION**

After several reports have presented information suggesting that animals may serve as reservoirs for MRSA infection of humans, the occurrence of...
MRSA in dogs have documented more frequent in recent years 
1,11,12,27-29. However, very little is known about the clinical significance of MRS for dogs 10,11,37-39. 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 7,20,26,30,31,34. In dogs, although S. intermedius is the most prevalent pathogenic Staphylococcus spp., several researchers 1,8,21,32 have 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. 26 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 1,2,11,14 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 10,20,34,42. Thus, it has been stated that CoNS were isolated both from healthy dogs 10,16,12 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 14,21,26. 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 femA 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 8,12,26-30,31,34 and methicillin-resistant S. intermedius 10,11,39,41 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 6,9,22,39. Also, our findings are supported by those of Gortel et al. 10, Van Duijkeren et al. 19, and Baptiste et al. 29, 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 predominant 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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Methicillin Resistance among Coagulase-positive Staphylococcus aureus


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