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RESEARCH ARTICLE

Prevalence of Methicillin-Resistant Staphylococci in Dogs [1]

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Summary

The aim of the study was to investigate the occurence and species distribution of methicillin resistant staphylococci (MRS) in the nasal cavity of dogs. Nasal swabs were collected from 162 dogs entering private veterinary clinics in Hatay. Methicillin resistance was detected onto mannitol salt agar containing 2 µg/ml oxacillin and confirmed by *mec*A Polymerase Chain Reaction (PCR). Bacterial identification was done using 16S rRNA sequencing. Staphylococcal cassette chromosome *mec* (SCC*mec*) typing of these isolates were determined by multiplex PCR. Antimicrobial susceptibility testing were performed disk diffusion method and antimicrobial resistance genes were determined by PCR. Methicillin-resistant coagulase negative staphylococci (MRCNS) harbouring *mec*A were isolated from 15.4% (25/162) of dogs. The species identified were *S. epidermidis* (n=12), *S. lentus* (n=6), *S. hominis* (n=4), *S. warneri* (n=1), *S. arlettae* (n=1) and *S. haemolyticus* (n=1). *mec*A-mediated methicillin resistance in *S. arlettae* was described for the first time. Methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin resistant *Staphylococcus pseudintermedius* (MRSP) were not detected. SCC*mec* type I, II, III and IV were identified in 1, 10, 9 and 5 MRS isolates, respectively. The results indicate that continuous surveillance is necessary to determine the emergence of MRS including MRSA.

Keywords: *Dog, Methicillin resistance, Staphylococci*

Köpeklerde Metisilin Dirençli Stafilokokların Prevalansı

Özet

Bu çalışmanın amacı, köpeklerin nazal mukozalarında metisilin dirençli stafilokokların (MRS) varlığınının ve tür dağılımının belirlenmesidir. Bu amaçla, Hatay'da özel veteriner kliniklerine getirilen 162 köpekten nazal svablar alındı. Metisilin direncinin belirlenmesinde 2 µg/ml oksasillin içeren mannitollü tuzlu agar kullanıldı. Bakteriyel identifikasyon 16S rRNA dizi analizi ile gerçekleştirildi. Stafilokokal kromozomal kaset tiplendirmesi (SCC*mec*) için multipleks polimeraz zincir reaksiyonu (mPZR) yapıldı. Antimikrobiyal duyarlılıkları disk diffuzyon yöntemi ile ve antimikrobiyal direnç genleri PZR ile incelendi. Köpeklerin %15.42'ünden (25/162) *mec*A geni taşıyan MRS izole edildi. Yirmibeş MRS izolatı *S. epidermidis* (n=12), *S. lentus* (n=6), *S. hominis* (n=4), *S. warneri* (n=1), *S. arlettae* (n=1) ve *S. haemolyticus* (n=1) olarak identifiye edildi. *S. arlettae*'da *mec*A geni ilk kez belirlendi. Metisilin dirençli *S. aureus* (MRSA) ve *S. pseudintermedius* (MRSP) izole edilmedi. SCC*mec* tip I, II, III ve IV sırasıyla 1, 10, 9 and 5 MRS izolatında belirlendi. Sonuçlar, MRSA dahil MRS suşlarının ortaya çıkışını belirlemek için sürekli surveyansın gerekli olduğunu işaret etmektedir.

Anahtar sözcükler: *Köpek, Metisilin Direnci, Stafilokok*

INTRODUCTION

Emergence of methicillin resistant staphylococci (MRS), particularly methicillin resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus pseudointermedius* (MRSP) in pet animals, is public and animal health concern due to zoonotic transmission of these multidrug resistant bacteria. MRSA strains found in dogs in various countries have been shown to be same clones isolated from humans in the region [1]. However, methicillin resistance among coagulase

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negative staphylococci (CNS) have been reported with increased frequency in human and veterinary medicine [2-6]. As there is limited data on the presence of MRSA, MRSP and other MRS in dog population in Turkey. This study investigated the prevalence of MRS carriage among dogs presenting private veterinary clinics in Hatay, Turkey.

MATERIAL and METHODS

Sample Collection

From December 2008 to June 2009, nasal swabs were obtained from 162 dog attending private veterinary clinics in Hatay, Turkey. This study was approved by the Animal Ethical Committee of Mustafa Kemal University (2008/78).

Sample Analysis

The swabs were placed in enrichment broth containing 10 g/l mannitol, 65 g/l sodium chloride, 2.5 g/l yeast extract and 10 g/l tryptone containing 2 µg/ml oxacillin and incubated at 35°C for 24 h. Subsequently, 10 µl inoculum was spread onto Mannitol Salt Agar containing 2 µg/ml oxacillin as above and incubated 35°C for 24-48 h. A single presumptive methicillin resistant staphylococcal colony was selected and identified phenotypically on the genus level by conventional biochemical tests.

DNA Extraction

Genomic DNA from individual pure cultures of MRCNS isolates were extracted with InstaGene matrix (Bio-Rad Laboratories, Canada) according to the manufacturer's instructions.

Identification and Characterisation of MRS Isolates

For the detection of *mec*A (methicillin resistance) gene, the oligonucleotide primers and PCR conditions used for this study were performed as reported previously by Oliveira and de Lencastre [7]. 16S rRNA gene amplification and sequence analysis were performed as described previously [8,9]. Thus, a large, 1371 bp fragment encoding 16S rRNA gene was amplified and subjected to sequence analysis for species discrimination. For the detection of 16S20 and 16S1390 universal rRNA, primers 5'- AGA GTT TGA TCC TGG CTC AG -3' and 5'- GAC GGG CGG TGT GTA CAA -3'were used as the forward and the reverse primer, respectively [10,11]. Nucleotide sequences were compared with the published sequences on National Center of Biotechnology Information (available online at http://www.ncbi.nlm.nih.gov), and sequences showing highest similarity score (>97%) to a type strain was considered as species identity.

SCCmec Typing

SCC*mec* types (I-IV) of the isolates were determined using methods and primers described by Oliveira and de Lencastre [7]. For the detection of *mec*A (methicillin resistance) gene and SCC*mec* typing, methicillin susceptible (*Staphylococcus aureus* ATCC 29213) and methicillin resistanat (*S. aureus* HPV107, *S. aureus* BK2464, *S. aureus* HUSA304, *S. aureus* GRE14) reference strains used as negative and positive control in PCR, respectively. Visualization of PCR products was performed on 1.5% agarose gel stained with ethidium bromide.

Animicrobial Susceptibility Testing

Antimicrobial susceptibility testing of MRS strains was performed according to the guideline of Clinical and Laboratory Standards Institute (CLSI) [12] using the following antimicrobial disks: erythromycin (15 µg), trimethoprim-sulfamethoxazole (1.25 µg/23.75 µg), vancomycin (30 µg), gentamicin (10 µg), quinopristin-dalfopristin (15 µg), ciprofloxacin (5 µg), mupirocin (5 µg), fusidic acid (10 µg), rifampicin (5 µg), amoxicillin-clavulanic acid (20 µg/10 µg), clindamycin (2 µg) and tetracycline (30 µg). Since standardized CLSI breakpoint for mupirocin and fusidic acid are not available, the disk diffusion testing of these antibiotics was performed as previously reported [13,14].

Determination of Antimicrobial Resistance Genes

PCR assays for the resistance genes *erm*A, *erm*B, *erm*C, *msr*A, *mph*C, *lun*A, *aac(6')/aph(2")*, *aph(3')-IIIa*, *ant(4')-Ia*, *tet*K, *tet*M, *ileS-2*, *fus*B, *fus*C was performed as previously reported [15-21].

RESULTS

Identification, Characterisation and SCCmec Types of MRS Isolates

MRS was isolated from 25 dogs (15.4%). Identification of isolates was done by sequencing a 1371 bp size PCR product by using universal 16S rRNA primers *(Fig. 1)*. 16S rRNA sequencing of isolates revealed the occurence of seven species: *S. epidermidis* (n=12), *S. lentus* (n=6), *S. hominis* (n=4), *S. warneri* (n=1), *S. arlettae* (n=1) and *S. haemolyticus* (n=1) *(Table 1)*. No dogs were colonized with MRSA and MRSP. The most prevalent SCC*mec* type were SCC*mec* II (40%), followed by SCC*mec* III (36%), SCC*mec* IV (20%), and SCC*mec* I (4%) *(Fig. 2, 3)*. While 20 isolates including type I, II and III were defined as hospital acquired methicillin resistant staphylococci (HA-MRS), 5 isolates including type IV were community acquired methicillin resistant staphylococci (CA-MRS).

Antimicrobial Susceptibility Testing

Ninety-two percent of isolates displayed resistance to at least one antimicrobial agent. Many MRCNS isolates were frequently resistant to erythromycin (14/25, 56%), tetracycline (13/25, 52%) and clindamycin (8/25, 32%). In addition, six (24%) of the isolates were resistant to ciprofloxacin and trimethoprim-sulfamethoxazole, five (20%) to gentamicin

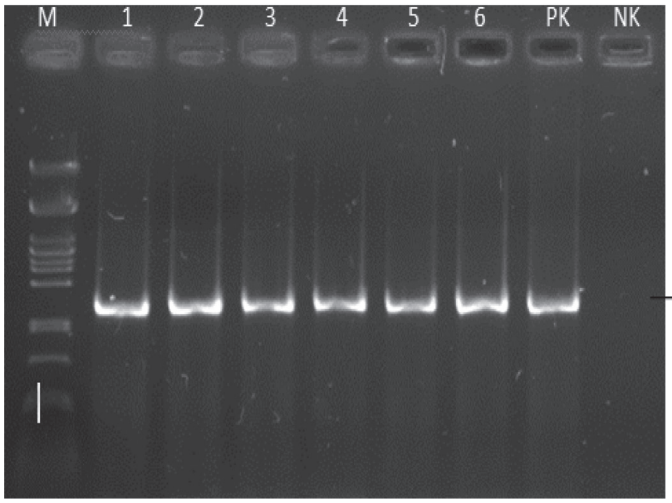

Fig 1. PCR performed by using 16S rRNA universal primers. M: Marker (Lambda phage DNA restricted with *Pst*I enzyme) 1-6: PCR performed by using isolated microorganism's DNA. PC: Positive control (*S. aureus* HPV107), NC: Negative control (master mix without DNA)

Şekil 1. 16S universal primerleri kullanılarak gerçekleştirilen PZR. M: Marker (*Pst*I enzimi ile kesilmiş lambda faj DNA'sı). PZR. 1-6: *Staphylococcus* izolatları, PK: Pozitif kontrol (*S. aureus* HPV107), NK: Negatif kontrol (DNA'sız master miks)

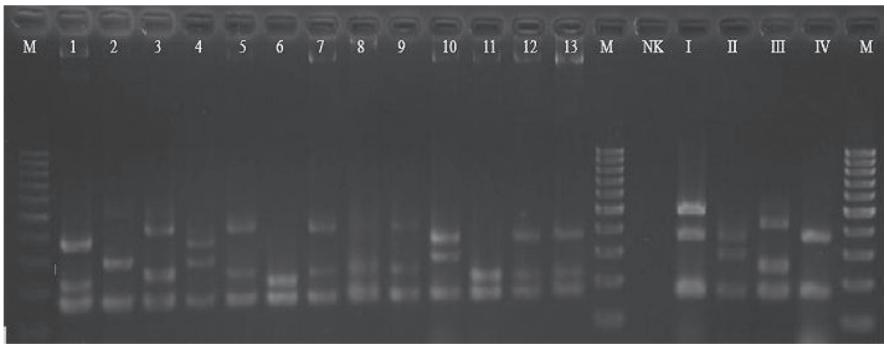

Fig 2. SCC*mec* types determined in MRS isolates. Lane M: 100 bp molecular marker. Lane 1-13: SCC*mec* types belong to different MRS isolates, Lane NC: Negative control (master mix without DNA), Lane I: *S. aureus* HPV107 (SCC*mec* type I), Lane II: *S. aureus* BK2464 (SCC*mec* type II), Lane III: *S.aureus* HUSA304 (SCC*mec* type III), Lane IV: *S. aureus* GRE14 (SCC*mec* type IV)

Şekil 2. MRS izolatlarında belirlenen SCC*mec* tipleri. M: 100 bp moleküler marker, 1-13: Farklı MRS izolatlarına ait SCC*mec* tipleri. NK: Negatif kontrol, I: *S. aureus* HPV107 (SCC*mec* tip I), II: *S. aureus* BK2464 (SCC*mec* tip II), III: *S. aureus* HUSA304 (SCC*mec* tip III), IV: *S. aureus* GRE14 (SCC*mec* tip IV)

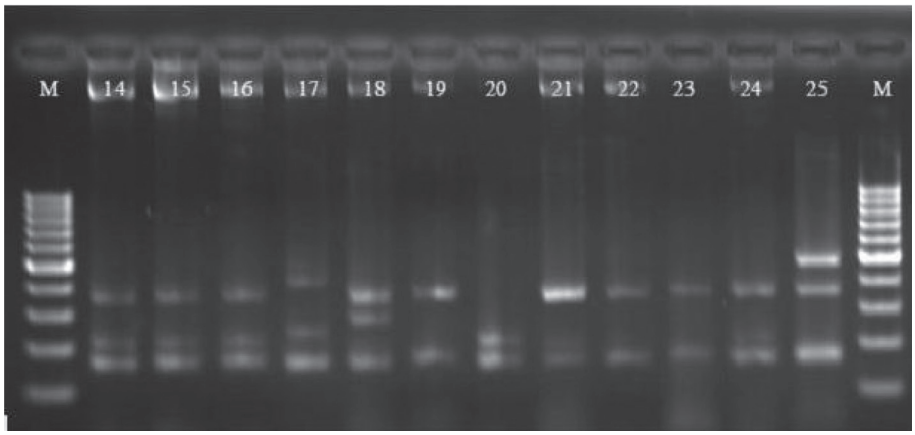

Fig 3. SCC*mec* types determined in MRS isolates. Lane M: 100 bp molecular marker. Lane 14-25: SCC*mec* types belong to different MRS isolates

Şekil 3. MRS izolatlarında belirlenen SCC*mec* tipleri. M: 100 bp moleküler marker. 14-15: Farklı MRS izolatlarına ait SCC*mec* tipleri

and mupirocin, three (12%) to rifampicin and one (4%) to quinopristin-dalfopristin, fusidic acid, and amoxicillin-clavulanic acid. But, all MRCNS isolates were found to be susceptible to vancomycin. All *S. hominis*, *S. warneri* and *S. haemolyticus* isolates displayed multiple antimicrobial resistance *(Table 1)*.

Prevalence of Resistance Genes

The *mec*A was detected in all strains. Of the 14 erythromycin-resistant (ER) isolates, 12 (85.7%) were positive for *erm*C, followed by *erm*B (9/14; 64.3%), *mph*C (9/14; 64.3%), *msr*A (7/14; 50.0%) and *erm*A (1/14, 7.1%). The *tet*K was the most prevalent gene among tetracycline resistant isolates, detected alone in 8 (61.5%) isolates, in combination with *tet*M in 3 (42.8%) isolates. The *tet*M was detected in two (15.4%) isolates. Among aminoglycoside-resistant isolates, *aac(6')/aph(2")* was detected in three (60%) strains, *aph(3')-IIIa* and *ant(4')-Ia* in one strain, and *ant(4')-Ia* in one isolate. Eight clindamycin resistant isolates were positive for *lnu*A gene. While only three isolates carried *ileS-2* gene among five mupirocin resistant isolates, *fus*B and *fus*C genes were not detected in one fusidic acid resistant isolate *(Table 1)*.

Table 1. *Antimicrobial resistance phenotypes, genotypes and SCCmec types of methicillin resistant coagulase negative staphylococci isolated from dogs*
Tablo 1. *Köpeklerden izole edilen metisilin dirençli koagülaz negatif stafilokokların antimikrobiyal direnç fenotipleri, genotipleri ve SCCmec tipleri*

MRCoNS Species	Phenotype*	Genotype	SCC*mec*Type
S. epidermidis	OXA, E	*mec*A, *erm*B, *erm*C, *mph*C	I
S. epidermidis	OXA, E, MUP, CIP	*mec*A, *erm*B	IV
S.epidermidis	OXA, TE, MUP	*mec*A, *tet*K, *ileS-2R*	IV
S. epidermidis	OXA	*mec*A	III
S.epidermidis	OXA, TE, E, DA, CIP	*mec*A, *tet*K, *erm*C, *msr*A, *mph*C, *lnu*A	II
S. epidermidis	OXA, SXT	*mec*A	IV
S. epidermidis	OXA, TE, E, SXT	*mec*A, *tet*K, *erm*C, *msr*A, *mph*C	IV
S. epidermidis	OXA, E, MUP	*mec*A, *erm*B, *erm*C, *msr*A, *ileS-2R*	III
S. epidermidis	OXA, TE, E, DA, FD	*mec*A, *tet*K, *erm*B, *erm*C, *mph*C	II
S. epidermidis	OXA, E, CN, DA, QD, MUP	*mec*A, *erm*B, *erm*C, *msr*A, *mph*C, *aac(6')/aph(2'')*, *lnu*A, *ileS-2R*	II
S. epidermidis	OXA	*mec*A	III
S. epidermidis	OXA, TE, E, DA, CIP	*mec*A, *tet*K, *erm*C, *msr*A, *mph*C, *lnu*A	II
S. lentus	OXA, TE	*mec*A, *tet*K, *tet*M	II
S. lentus	OXA, TE, CN, RD	*mec*A, *tet*M, *aac(6')/aph(2'')*	II
S. lentus	OXA, TE, DA	*mec*A, *tet*K, *tet*M, *lnu*A	II
S. lentus	OXA, TE, E, DA, SXT, CIP	*mec*A, *tet*K, *tet*M, *lnu*A, *erm*A, *erm*B, *erm*C, *mph*C	III
S. lentus	OXA, AMC, SXT, CIP	*mec*A	II
S. lentus	OXA, TE	*mec*A, *tet*K	IV
S. hominis	OXA, TE, CN, RD	*mec*A, *tet*K, *aac(6')/aph(2'')*	II
S. hominis	OXA, TE, E, CN, DA	*mec*A, *tet*M, *lnu*A, *erm*B, *erm*C, *msr*A, *mph*C, *aph(3')-IIIa*, *ant(4')-Ia*	III
S. hominis	OXA, E, MUP	*mec*A, *erm*C	III
S. hominis	OXA, TE, E, SXT	*mec*A, *tet*K, *erm*B, *mph*C	III
S. warneri	OXA, E, DA, CN	*mec*A, *lnu*A, *erm*B, *erm*C, *msr*A, *ant(4')-Ia*	III
S. arlettae	OXA, SXT	*mec*A	II
S. haemolyticus	OXA, E, RD, CIP	*mec*A, *erm*C	III

**** OXA:*** *oxacillin,* ***E:*** *erythromycin,* ***SXT:*** *trimethoprim-sulfamethoxazole,* ***QD:*** *quinopristin-dalfopristin,* ***CN:*** *gentamicin,* ***CIP:*** *ciprofloxacin;* ***MUP:*** *mupirocin,* ***FD:*** *fusidic acid,* ***RA:*** *rifampicin,* ***AMC:*** *amoxicillin-clavulanic acid,* ***DA:*** *clindamycin,* ***TE:*** *tetracycline*

The most prevalent SCC*mec* type were SCC*mec* II (40%), followed by SCC*mec* III (36%), SCC*mec* V (20%), and SCC*mec* I (4%) *(Fig. 2, 3)*.

DISCUSSION

Considering the high zoonotic potential of MRSA and MRSP, it is encouraging that MRSA and MRSP were not isolated from any dogs sampled in this study. This indicates that these agents have a very low in the total population of dogs admitted to clinics. Similar results have been reported in Turkey and Denmark [4,6].

CNS are recognised as a major cause of nosocomial infections, especially in immunocompromised patients [2]. An increase of MRCNS strains was reported from 38% in 1996 to 67.5% in 2007 in Turkey [23]. Although, importance of CNS in veterinary medicine or potential for zoonotic infection is not well known. In recent years, CNS has steadily gained importance as veterinary pathogens and implicated in mastitis, pyoderma, cystitis, arthritis and respiratory system infections in various animal species [8,9,24,25].

The most prevalent SCC*mec* types were II (40%, 10/25) and III (36%, 9/25), identified among all MRCNS. Type IV is predominant among *S. epidermidis* isolates. SCC*mec* type IV was more frequently acquired by *S. epidermidis*, which is in accordance with the enhanced mobility of this type of SCC*mec*. A majority of hospital-acquired MRSA (HA-MRSA) isolates harbor SCC*mec* type I-III [26], SCC*mec* type III were found to be more prevalent among human MRSA strains with a prevalence rate of 82.1% in Turkey [27]. Dominance of HA-MRSA SCC*mec* type II and III indicate that dogs are a large reservoir of SCC*mec* in MRCNS. It is reasonable to assume that CoNS of dog origin share a common pool of SCC*mec* with MRSA and thus pose a potential threat to public and animal health.

Among 25 MRCNS isolated, *S. epidermidis*, *S. lentus* and *S. hominis* were most prevalent species. To the best of our knowledge, this is the first report of *mec*A-mediated

methicillin resistance in *S. arlettae* in dogs. *S. arlettae* is one of the CoNS isolated from the skin of mammals and poultry [28]. Bagcigil et al.[4] reported *S. epidermidis* (n=7) and S. *haemolyticus* (n=3) as more prevalent species in dogs in Denmark. Another study carried out in Turkey, *S. hominis* was found to be the more prevalent among MRCNS in dogs [6]. Although no information is available on the frequency of nosocomial pathogens in veterinary hospitals, some species, mainly *S. epidermidis*, *S. haemolyticus*, *S. hominis* have been isolated from nosocomial infections in Turkey [23,29].

Methicillin resistant strains have high rates of resistance to other classes of antimicrobials than methicillin susceptible strains [2]. In this study, MRCNS strains were resistant to clinically relevant antimicrobial drugs such as mupirocin, fusidic acid, quinopristin-dalfopristin, rifampicin in various levels. These findings confirm that MRS may pose a major therapeutic challenge for veterinarians due to limited choise of antimicrobials. Taken into consideration of multiple resistance, antimicrobial selective pressure is likely to play a key role in the emergence and spread of MRCNS among dog population.

All except two MRCNS isolates carried more than one antimicrobial resistance gene. In particular, one *S. hominis* isolate carried nine resistance genes that confer resistance to five antimicrobials. Hanssen and Sollid [30] reported that resistant strains of CNS might serve as pool of antimicrobial resistance genes. Because majority of resistance determinants carried by mobile genetic elements, and this favors transfer of resistance genes within and across bacteriel species and even across genus borders

In conclusion, the results indicate that MRCNS are common in dogs in Turkey. Therefore, the resistance trends observed among staphylococci isolated from the nasal cavity of dogs seem to reflect the national and local patterns of antimicrobial usage in this animal species. However, further studies based on larger and more representative study populations are needed to determine the true prevalence of these agents.

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