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RESEARCH ARTICLE

Methicillin Resistance Profile and Molecular Typing of *Staphylococcus aureus* Strains Isolated from Noses of the Healthy Dogs [1]

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Summary

This study was conducted to determine the profile of methicillin resistance of *Staphylococcus aureus* strains isolated from noses of healthy dogs in Samsun and to investigate the coagulase *(coa)* and protein A *(spa)* gene polymorphisms of these isolates. A total of 80 isolates were identified as coagulase positive *S. aureus* phenotypically and genotypically. All of the isolates were found to be methicillin sensitive determined by agar disc diffusion method. However, in multiplex PCR performed to detect mecA and fem genes, 3 (3.75%) isolates were found to have mecA gene and none were fem positive. Amplification of the coa and spa genes from 80 *S. aureus* isolates produced 9 and 3 different PCR products, respectively. All 3 methicillin resistant isolates identified by multiplex PCR were observed in the same coa and spa groups. It was concluded that various coagulase types and more than one spa types of *S. aureus* are present in the region where the study was performed and MRSA isolates were included in unique coa and spa groups. Also, the MRSA colonization rate was considered to be high and may pose a risk for human health.

Keywords: *Coa, Dog, MecA, Nasal carriage, Staphylococcus aureus, Spa*

Sağlıklı Köpeklerin Burunlarından İzole Edilen *Staphylococcus aureus* Suşlarının Metisilin Direnç Profili ve Moleküler Tiplendirilmesi

Özet

Bu çalışma, Samsun İli'ndeki sağlıklı köpeklerin burunlarından izole edilen *Staphylococcus aureus* suşlarının metisilin direnç profilini belirlemek ve bu izolatların koagulaz *(coa)* ve protein A *(spa)* gen polimorfizmlerini araştırmak üzere gerçekleştirildi. Toplam 80 izolat, fenotipik ve genotipik olarak koagulaz pozitif *S. aureus* olarak identifiye edildi. İzolatların tümü agar disk difüzyon yöntemi ile metisilin duyarlı bulundu. Bununla birlikte, mecA ve fem genlerini belirlemeye yönelik yapılan multipleks PCR'da 3 (%3.75) izolatın mecA genine sahip olduğu bulunurken, suşların hiçbiri fem pozitif değildi. Seksen *S. aureus* izolatından coa ve spa genlerinin amplifikasyonu sonucunda sırasıyla 9 ve 3 farklı PCR ürünü elde edildi. Multipleks PCR ile metisilin dirençli *S. aureus* olarak identifiye edilen 3 izolatın da aynı koagulaz ve spa grubunda olduğu belirlendi. Çalışılan bölgede, çok sayıda coa genotipine ve birden fazla spa grubuna sahip *S. aureus* suşlarının bulunduğu ve MRSA izolatlarının tek bir coa ve spa grubuna dahil olduğu sonucu çıkarıldı. Bu sonuçlar sağlıklı köpeklerde MRSA kolonizasyon oranının yüksek olduğunu ve insan sağlığı için risk teşkil edebileceğini gösterdi.

Anahtar sözcükler: *Burun taşıyıcılığı, Coa, Köpek, MecA, Staphylococcus aureus, Spa*

INTRODUCTION

Staphylococci is common type of bacteria that live on the skin and mucous membranes of humans and animals. In the hospital and community settings, treatment of infections by this pathogen has become a serious problem because of acquisition of resistance to several antimicrobial agents. Resistance to methicillin

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is considered of great importance since oxacillin is the drug of choice for the treatment of *S. aureus* infections [1]. Acquisition of the *mecA* gene, encodes for the penicillin binding protein PBP2a, confers virtually complete resistance to all beta-lactam antibiotics including the semisynthetic penicillins (e.g., methicillin, oxacillin). Methicillin-resistant strains of *S. aureus* (MRSA) have become a concern in veterinary medicine. Asymptomatic colonization with MRSA, including nasal carriage, has been reported in animals [2,3]. Case reports of human infection or colonization from companion animals indicate that animals act as reservoirs for MRSA transmission [3]. Through investigation of possible zoonotic infections to establish linkage is encouraged. There are concerns about MRSA as a possible zoonosis. Although it has not yet been determined whether animals are an important primary source of MRSA infections for humans or if most animals are colonized after contact with human carriers, both human-to-animal and animal-to-human transmission are known to be possible [2]. MRSA is of particular concern and there have been increasing reports of infections in dogs and cats both in veterinary hospitals and in the community [4]. Concern about MRSA in the community has led to recommendations for surveillance of carriage levels in healthy dogs [3]. Medical and veterinary staff should appreciate that animals can carry MRSA, cooperate in eliminating infections and monitor animals in medical environments.

PCR is accepted as *"gold standard"* for the detection of the genotypic characteristics of pathogens [5,6]. Likewise, the detection of the presence of mecA gene by PCR is accepted as *"gold standard"* for methicillin resistance of staphylococci. Detection of methicillin resistance is influenced by several factors as mec regulatory genes, β-lactamase regulatory genes and fem genes [7]. *S. aureus*, like other bacterial species, has several subtypes which are related with the presence and expression of the virulence genes such as *coa* and *spa* genes. Accurate identification of *"successful"* clones with enhanced virulence or increased ability to spread epidemically is essential [8,9]. The molecular typing systems for *S. aureus* have been substituted for the conventional typing methods. These have several advantages such as high performance (typeability, reproducibility, stability, and discriminatory power) and convenience (rapidity, accessibility, ease of use, and ease of interpretation) over the traditional methods [9]. Genes coding for two specific proteins, coagulase (coa) and protein A *(spa)* have been the most widely used markers for molecular typing as they contain highly polymorphic repeat units [10].

There are limited number of reports on MRSA infections in pet animals and therefore few typing studies have been reported [11,12]. No report could be found concerning neither about carriage of MRSA nor typing of *S. aureus* in dogs in Turkey. Considering the rapid increasing MRSA in the community and the fact that pet animals, especially dogs, are in close contact with their owners, the risk of transmission of such bacteria between animals and humans must be considered. In this study, the detection of nasal carriage of methicillin resistant *S. aureus* in nasal microflora of dogs and molecular typing of the strains identified as *S. aureus* by PCR-based methods targeting coa and spa gen polymorphisms were aimed.

MATERIAL and METHODS

Specimen Collection

Total of 390 specimens was collected using a sterile cotton swap from nares of the dogs which came to veterinary clinics for a routine examination and found as healthy in Samsun during 2007 and 2008. The swabs were transferred to a transport medium (Stuart's Medium) and transported to laboratory at 2-8°C within 8 h.

Culture Conditions

Swabs were inoculated into MRSA enrichment broth (2.5% NaCl, trypticase soy broth), vortexed for 10 seconds and incubated at 37°C for 48 h. Then, inoculums were plated onto Baird Parker Agar (BPA) with 6 µg/ml oxacillin (Sigma) and BPA without oxacillin, separately and incubated at 37°C for 48 h. Methicillin-sensitive *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) strains were detected according to growing *S. aureus* colonies on BPA with or without oxacillin. Growing on both mediums was the indicator of the presence of methicillin resistance.

Identification and methicillin resistance of isolates

S. aureus was identified as described by Murray [13]. To detect MRSA, Kirby-Bauer Disc Diffusion Method was utilized with 5 µg oxacillin discs on Mueller-Hinton Agar according to the NCCLS recommendation [14].

DNA extraction

Bacterial DNAs were extracted with a commercial

extraction kit (Omega Bio-tek, Inc.) based on the filtration through a spin colon according to the manufacturer's instructions. Extracted DNA (100 µl) was kept frozen at -20°C until the further analyses conducted.

Genotypic identification and mecA presence of S. aureus

A multiplex PCR (multiplex-1) was performed for the identification of *S. aureus* genotypically [15]. To determine methicillin resistance of *S. aureus* strains, a multiplex PCR (multiplex-2) targeted *mec*A and fem genes was performed [16]. The primers used for detection of mec and fem genes are listed in *Table 1*.

Coagulase gene (coa) and protein A gene (spa) typing

The primers *(Table 1)* and PCR conditions were followed as previously described [17,18].

Table 1. *Primers used in this study*
Tablo 1. *Çalışmada kullanılan primerler*

Primer	Oligonucleotide sequence (5'- 3')
16S rRNA (f)	AACTCTGTTATTAGGGAAGAAGAACA
16S rRNA (r)	CCACCTTCCTCCGGTTTGTCA
nuc (f)	GCGATTGATGGTGATACGGTT
nuc (r)	AGCCAAGCCTTGACGAACTAAAGC
*mec*A (f)	CCTAGTAAAGCTCCGGAA
*mec*A (r)	CTAGTCCATTCGGTCCA
fem (f)	CTTACTTACTGCTGTACCTG
fem (r)	ATCTCGCTTGTTATGTGC
coa (f)	CGAGACCAAGATTCAACAAG
coa (r)	AAAGAAAACCACTCACATCA
spa (f)	TCAAGCACCAAAAGAGGAAGA
spa (r)	GTTTAACGACATGTACTCCGTTG

Typeability, discriminatory power, and reproducibility

The proportions of strains that can be assigned a type by typing methods used in the current study were calculated as previously described [19,20]. To test PCR (*coa* and *spa* typing) reproducibility by interassay analysis, 5 isolates were selected randomly and were tested for 5 consecutive days.

RESULTS

Identification of strains and determination of methicillin resistance

Among all 390 samples, 80 *S. aureus* were isolated from noses of healthy dogs and identified as coagulase positive *S. aureus* phenotypically by standard laboratory methods indicated above. All isolates were found to be methicillin-sensitive phenotypically by disc diffusion method. In multiplex-1 PCR performed to confirm *S. aureus* strains, all strains were positive for 16SrRNA and nuc gene. In multiplex-2 PCR performed to detect methicillin resistance genotypically, 3 (3.75%) of all *S. aureus* strains were found to be *mec*A positive and no strain was fem positive.

coa typing

Eighty *S. aureus* isolates from noses of dogs were evaluated for coagulase gene polymorphism by PCR and isolates produced one or more fragment sizes of PCR products. Strains were divided into 9 groups (C1-C9) based on the PCR product patterns. C1 was the predominant group consisting of 26 (32.5%) isolates. Strains in 8 groups (C1, 2, 3, 4, 5, 6, 8 and 9) produced a single DNA fragment by PCR with sizes ranging from 326 to 1,500 bp. Only three isolates belonging to C7 exhibited 2 PCR products, 437 bp and 697 bp. Coagulase gene typing results are summarized in *Table 2*.

spa typing

Eighty *S. aureus* isolates were typed based on differences in the Xr region of the *spa* gene encoding for protein A by PCR. Isolates were divided into 3 groups according to this typing system, where 3 PCR products of different sizes were observed following amplification of the *spa* gene. Seventy-one isolates (88.75%) were assigned to the S1 group which produced 310 bp of product. S2 and S3 groups included 3 (3.75%) and 6 (7.5%) isolates, respectively. The amplicon sizes of strains belonging to S2 and S3 were 270 bp and 290 bp, respectively. The results of *spa* typing by PCR are summarized in *Table 2*.

Table 2. *The results of coa and spa genotyping of S. aureus isolates*
Tablo 2. *S. aureus izolatlarının coa ve spa genotiplendirme sonuçları*

***coa* genotype**		***spa* genotype**		**n**	**%**
C1	470 bp	S1	310 bp	26	32.50
C2	810 bp	S1	310 bp	17	21.25
C3	490 bp	S1	310 bp	12	15.00
C4	521 bp	S1	310 bp	9	11.25
C5	570 bp	S1	310 bp	6	7.50
C6	326 bp	S3	290 bp	3	3.75
C7	437 and 697 bp	S2	270 bp	3	3.75
C8	710 bp	S3	290 bp	3	3.75
C9	1500 bp	S1	310 bp	1	1.25

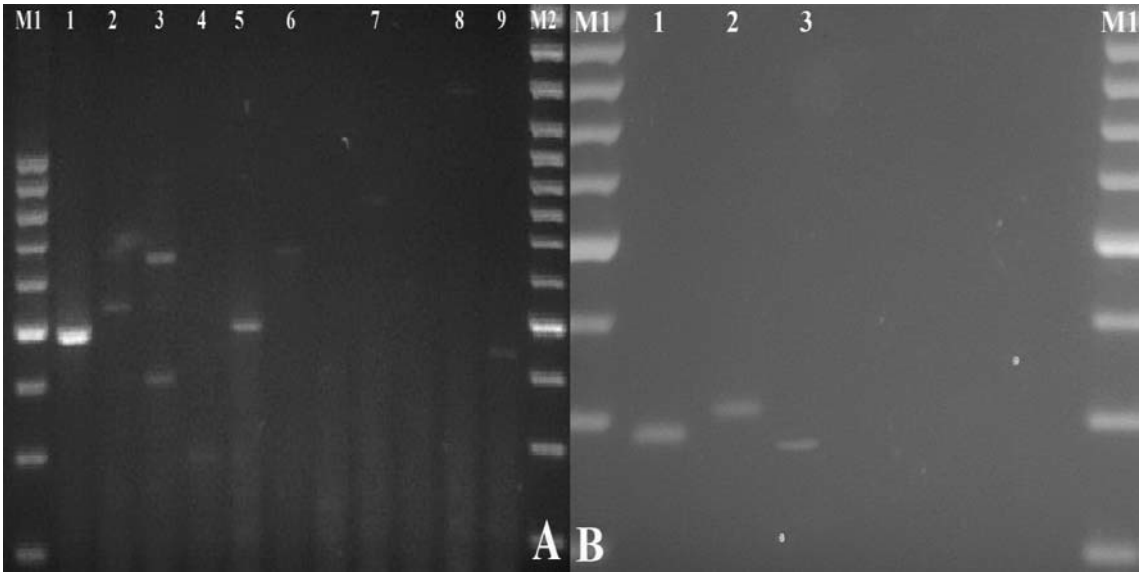

Fig 1. The genotypes of *S. aureus*. A) coa genotyping results. M1: Molecular weight marker (100-1031 bp) M2: Molecular weight marker (100-3000 bp) (Fermentas Life Sciences, Vilnius, Lithuania), Lanes: 1; 490 bp (C3), 2; 570 bp (C5), 3; 437 and 697 bp (C7), 4; 326 bp (C6), 5; 521 bp (C4), 6; 710 bp (C8), 7; 810 bp (C2), 8; 1500 bp (C9), 9; 470 bp (C1), B) spa genotyping results. Lanes: 1; 290 bp (S3), 2; 310 bp (S1), 3; 270 bp (S2)

Şekil 1. *S. aureus* genotipleri. A) coa genotiplendirme sonuçları. M1: Moleküler ağırlık standardı (100-1031 bp) M2: Moleküler ağırlık standardı (100-3000 bp) (Fermentas Life Sciences, Vilnius, Lithuania), Sütunlar: 1; 490 bp (C3), 2;570 bp (C5), 3; 437 ve 697 bp (C7), 4; 326 bp (C6), 5, 521 bp (C4), 6; 710 bp (C8), 7; 810 bp (C2), 8; 1500 bp (C9), 9; 470 bp (C1), B) spa genotiplendirme sonuçları. M1: Moleküler ağırlık standardı (100-1031 bp), Sütunlar: 1; 290 bp (S3), 2; 310 bp (S1), 3; 270 bp (S2)

Typeability, discriminatory power, and reproducibility

All *S. aureus* isolates produced amplified product(s) in both *coa*- and *spa*-PCR reflecting that these were typeable. Discriminatory indices for PCR-based *coa*- and *spa*-typing were 0.918 and 0.672, respectively. To determine the reproducibility of PCR for *coa* and *spa* typing by interassay analysis, 5 isolates were selected randomly and were tested for 5 consecutive days. The reproducibility of PCR-based typing methods (*coa*- and *spa*-typing) was 100%.

DISCUSSION

Staphylococcus aureus is a frequent cause of infections in both the community and hospital and is carried by approximately 25% of humans in their nasal cavities, which is a major reservoir of this pathogen. It has been shown that nasal carriers of *S. aureus* have an increased risk of acquiring an infection with this pathogen. The nose is the main ecological niche where *S. aureus* resides in human beings [3,21]. The frequency of *S. aureus* carriage on the skin and mucous membranes of dogs and cats is low. Isolates are generally recovered from less than 10% of samples [22]. Resistance to antibiotics, particularly to methicillin, complicates the treatment of *S. aureus* infections, MRSA is a worldwide particular concern and there have been increasing reports of infections in dogs and cats both in veterinary hospitals and in the community [2,4,23]. As in humans, MRSA can colonize the skin and nasal mucosa of healthy animals [23]. In this study, healthy dogs which examined for general control in veterinary clinics were sampled for nasal carriage of MRSA. Total of 80 (20.51%) *S. aureus* were isolated from 390 samples from noses of healthy dogs. This colonization rate was higher than the rate previously reported [22] but in a few surveys, carriage rates have been reported as high as 90% [2]. Most of the strains (96.25%) isolated in this study were MSSA and only 3 of them were *mec*A positive. Due to lack of information about the *S. aureus* colonization status in the owners of these animals, the source of these strains could not be confirmed. It was suspected that this high colonization rate may be due to transmission of *S. aureus* (MRSA or MSSA) from their owners which are carriers of *S. aureus*, as transmission of MSSA has been reported between owners and their pets [24]. In some cases, asymptomatic human carriers seem to have transmitted MRSA isolates to animals in veterinary hospitals [2]. Further studies in this area are required to investigate the transmission of *S. aureus* strains between dogs and their owners. Mucosal carriage of MRSA has been demonstrated in dogs [25-27] but

few data exist on the prevalence of MRSA in these animals. Abbott et al.[28] in Ireland have been reported that the prevalence of MRSA colonization was 0.6% in non-clinically infected dogs upon admission to veterinary clinics and they have also detected the prevalence of MRSA as 0.9% non-clinically infected dogs upon admission to a veterinary referral hospital. In another study [12], mucosal MRSA carriage in dogs has been found as 9%. In our study, the prevalence of MRSA in healthy dogs was found to be 3.75%. Although a few comparable data are available for dogs, this rate seems to be high. Comparison of MRSA carriage rates reported by different studies is problematic since various sampling strategies and isolation methods can be used for assessing staphylococcal carriage [12]. It was reported that colonized or infected animals can serve as reservoirs for disease both in other animals and humans [2]. It was considered that the high prevalence of MRSA found in this study may entail a risk for human infections.

Three *mec*A positive *S. aureus* strains identified in this study did not present methicillin resistant phenotype in agar disc diffusion test. Phenotypic expression of resistance can be effected from various factors related to growth conditions such as temperature and osmolarity of medium. Therefore some drawbacks may be occurred for making susceptibility testing of MRSA by standard microbiological methods [7]. Only rare isolates that carry *mec*A are phenotypically susceptible to methicillin. These isolates are considered to express heteroresistant trait and show high resistance to methicillin. Therefore, this type of isolates may lead to the failure of therapy [29,30]. It was concluded that in detection of MRSA, PCR targeting mecA gene must be conducted together with susceptibility testing in order to prevent the wrong diagnosis for methicillin resistance.

It has been reported that molecular typing of bacterial isolates on the basis of variations in the chromosomal DNA structure [31,32]. It is considered that among the methods for molecular typing of *S. aureus* isolates, pulsed-field gel electrophoresis (PFGE) is gold standard. PCR-based methods are easier, faster and less expensive than that of PFGE [9]. In this study, *S. aureus* isolates mostly identified as MSSA (96.25%) were typed genotypically by PCR based on polymorphisms in *coa*- and *spa* genes. Isolates were divided into 9 *coa*-types in this study, which indicate considerable heterogeneity in the *coa* gene of *S. aureus* isolated from clinically healthy dogs. Amplification of spa gene produced 3 different products and isolates divided into three spa groups. Most of the isolates (92%) were assigned to S1. MRSA isolates in this study were assigned to S2 group which was less common spa type. The pre-dominant *coa*-type was C1 (32.5%) and a single DNA fragment, 470 bp PCR product was observed in these isolates. In this study, double-bands were detected in 3 *coa*-positive isolates assigned to C7 group and these three isolates were *mec*A positive. The presences of double-banded products have been explained with the existence of more than one allelic form of coagulase gene [32]. MRSA isolates assigned to C7 were included in SP2 group according to *spa* polymorphism-based PCR. Discriminatory index (DI) value of 1.0 would indicate that a typing method was able to distinguish each member of a strain population from all other members of that population. Conversely, a DI of 0.0 would indicate that all members of a strain population were of an identical type [33]. In the current study, the DI values of *coa*-PCR typing and *spa*-PCR typing were 0.918 and 0.672, respectively. So, *coa*-PCR typing was more discriminative than *spa*-PCR typing.

Concern about MRSA in the community has led to recommendations for surveillance of carriage levels in healthy dogs [3]. Medical and veterinary staff should take a consideration that animals can carry MRSA, cooperate in eliminating infections and monitor animals in medical environments. Veterinary clinics should implement guidelines for dealing with MRSA [2]. It has been shown that even apparently healthy animals may be MRSA reservoirs, and therefore may pose a risk to their handlers. Ongoing MRSA surveillance in animals for both colonization and infection is necessary in order to clarify the epidemiology of the transmitted strains as well as develop measures to reduce transmission [7]. Although screening of all animals which are admitted to a veterinary clinic for controlling of the MRSA carriage does not seem to be practical, considering the relatively high carriage rate of MRSA in healthy dogs like in this study, it is considered that the animals should be examined for colonization of MRSA and tried to eliminate MRSA using some anti-microbials. In conclusion, the determining of the nasal carriage of different genotypes of *S. aureus* in healthy dogs was considered as an important finding for both the characterization of nasal isolates of *S. aureus* and molecular epidemiological researches. Further studies using more isolates from both humans and animals should be conducted to assess the similarity of these isolates with each other and transmission between human and animals.

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