Antimicrobial Susceptibility Profiles and Coagulase Gene Polymorphism of *Staphylococcus aureus* Isolated from Bovine Subclinical Mastitis [1] [2]

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Abstract

The purpose of the study was to isolate *Staphylococcus aureus* from bovine subclinical mastitis, determine their antibiotic susceptibilities and investigate the coagulase gene polymorphism by using a PCR-based restriction fragment length polymorphism (RFLP) method. Milk samples from 463 CMT positive udders from 237 cows cultured. The antimicrobial susceptibility of the isolates were determined by disc diffusion method. A total of 82 out of the 83 isolates (98.8%) were found to be resistant at least one out of the 16 antibiotics studied. In this experiment 53 isolates (63.8%) were found to be resistant to penicillin; 52 (62.67%) to trimethoprim/sulphamethoxazole; 51 (61.5%) to ampicillin; 40 (48.2%) to erytromycin; 29 (34.9%) to tetracycline; 18 (21.6%) to ciprofloxacin, 16 (19.3%) to clindamycin, 13 (15.6%) to chloramphenicol; 8 (9.6%) to gentamicin; 5 (6.0%) to cefoxitin; 4 (4.9%) to vancomycin; 3 (3.6%) to cephalotin; 2 (2.4%) to nafcillin; one (1.2%) to oxacillin and one (1.2%) to furazolidon. No imipenem resistance was seen in the *S. aureus* isolates. The coagulase gen polymorphism were examined by PCR amplification of coagulase gene followed by Alu digestion of repeating 81 bp DNA sequences. After nested PCR, double bands were produced in 8 of the isolates while there were single band in remaining 75 isolates. Following Alu digestion, isolates that formed single band in length of approximately 300 bp showed 3 different groups.

Keywords: Bovine subclinical mastitis, *Staphylococcus aureus*, Antibiotic susceptibility, Coagulase gene polymorphism

Subklinik Mastitli İneklerden İzole Edilen *Staphylococcus aureus* İzolatlarının Antibiyotik Duyarlılık Profillerinin Çıkarılması ve Koagulaz Geni Polimorfizminde Göre Tiplendirilmesi

Özet

Bu çalışmanın amacı subklinik mastitli sığırlardan *Staphylococcus aureus*’u izole etmek, bunların antibiyotiklere duyarlılığını belirlemek ve bir PCR tabanlı restrikşyon fragment lengh polymorphism (RFLP) yöntemi kullanarak koagulaz gen polimorfizmini araştırmaktır. 463 sürt CMT pozitif olan 237 sığır memesinden süt örnekleri alınarak ekim yapılmıştır. İzolatların antimikrobiyel duyarlılığı disk difüzyon yöntemi ile belirlenmiştir. Toplam 83 izolatın 82’i (%98.8) uygulanan 16 antibiyotikten en az bir antibiyotikte dirençli bulundu. Bu çalışmada 53 izolat (%63.8) penilin, 52 izolat (%62.67) trimethoprin/sulphamethoxazole, 51 izolat (%61.5) ampsilin, 40 izolat (%48.2) eritromisine, 29 izolat (%35.0) tetraksilin, 18 izolat (%21.7) siproflosksazin, 16 izolat (%19.3) kontamisin, 13 izolat (%15.6) kloramfenikol; 8 izolat (%9.6) gentamisin, 5 izolat (%6.0) sefoksitine, 4 izolat (%4.9) vancomisin, 3 izolat (%3.6) sefoksitine, 2 izolat (%2.4) nafsiline ve 1 izolat (%1.2) izolat ise oksidasin ve furazolidona dirençli bulundu. *S. aureus* izolatlarındaki imipenem direncini görüldü; Koagulaz gen polimorfizmi koagulaz genin tekranılan 81 bp DNA dizisinin *Alu* sindirimi mutesekkib koagulaz genin amplifikasyonu ile incelenmiştir. Nester PCR’den sonra izolatların %8inde çift bant görülmiş kalan 75 izolatta ise tek bant vardi. Alu sindiriminin mutesekkibin yaklaşık 300 bp uzunlukta tek bant oluşturulan izolatlar 3 farklı grup göstermiştir.

Anahtar sözcükler: Siğir subklinik mastitis, *Staphylococcus aureus*, Antibiyotik duyarlığı, Koagulaz gen polimorfizmi

INTRODUCTION

Staphylococcus aureus has a wide range host spectrum and can cause serious infections by its methicillin resistant isolates. Long-term antibiotic usage is important in development of resistance against methicillin and other beta-lactam antibiotics. S. aureus is one of the most important pathogen for cattle mastitis and it is prevalent all around the world. Despite of strict control measures, control and eradication of S. aureus caused intramammary infections are quite difficult and continue as an economical problem. Antimicrobial therapy is one of the measures can be taken in order to control of staphylococcal mastitis. Detection of antibiotic susceptibilities of clinical isolates is necessary not only for treatment but also for preventing spread of resistant isolates. Regional evaluation of antibiotic susceptibilities of S. aureus may help veterinary surgeons.[1,2]. Although most of the current S. aureus isolates have different genotypic and phenotypic characteristics, few are known about geographical distribution of those isolates and types of the pathogens of the herd[3]. Previously, distinct classification methods such as phage typing had been applied to both human and cattle originated S. aureus isolates.[4,5]. Afterwards, methods such as plasmid analysis, ribotyping, pulsed-field gel electrophoresis, PCR- based fingerprinting, amplification of specific gene regions, and binary typing technics were started to be PCR- based fingerprinting, amplification of specific gene regions, and binary typing technics were started to be applied to both human and cattle originated S. aureus isolates. Afterwards, methods such as plasmid analysis, ribotyping, pulsed-field gel electrophoresis, PCR- based fingerprinting, amplification of specific gene regions, and binary typing technics were started to be applied.[5,6,7,8]. In recent years, there are some publications about genetic diversity of S. aureus isolates in Turkey recovered from subclinical cattle mastitis cases.[9,10]. The aim of this study was to evaluate the biochemical capacity of the antibiotic resistances of S. aureus isolates recovered from subclinical cattle mastitis cases in the Middle Western Anatolia and perform molecular typing on coagulase gene polymorphism.

MATERIAL and METHODS

**Bacteriological Studies**

Milk samples were collected from 16 different dairy farms located in four different districts of Middle Western Anatolia between January-June 2010. Milk samples were collected in the mid-lactation period. California Mastitis Test (CMT) positive 463 milk samples were collected from 237 cows. The samples were inoculated onto Nutrient agar supplemented with 7% sheep blood, incubated at 37°C for 24-48 h. Eighty three S. aureus has been isolated and identified by the conventional tests such as Oxidase, catalase and coagulase positive (slide and tube), susceptibility to furazolidone, hemolysis, pigment formation, O/F, Baird Parker Agar (BP), Egg yolk tellurit, Mannitol Salt Agar (MSA), DNase Agar.[14,15].

Gram positive cocci were further identified with conventional biochemical test and API Staph (Bio Merieux, France). The isolates were kept at -70°C in Trypticase Soy Broth (TSB) containing 15% glycerine in order to further use in molecular studies. Antimicrobial susceptibility tests of the isolates were performed in accordance with National Committee for Clinical Laboratory Standards-NCCLS.[16]. The isolates were tested against the following antibiotics: penicillin (10 IU), gentamicin (10 μg), vancomycin (30 μg), clindamycin (2 μg), trimethoprim/sulfamethoxazole (1.25 μg/23.75 μg), cefalotin (30 μg), imipenem (10 μg), nafcillin (1 μg), furazolidone (100 μg), ampicillin (10 μg), tetracycline (30 μg), oxacillin (1 μg), chloramphenicol (30 μg), cefoxitin (30 μg), erythromycin (15 μg) and ciprofloxacin (5 μg). S. aureus ATCC 25923 was used as control strain.[17]. Chi square test was used to evaluate the significance between antimicrobial sensitivities or resistances of S. aureus isolates.

**Molecular Studies**

DNA extractions from S. aureus isolates were performed by using genomic DNA purification kit (Bio Basic Inc., Toronto, Canada). In addition to the protocol, 11 U lyso-staphin was added during lysis phase. Multiplex PCR developed by Maes et al.[18], were performed for the confirmation of S. aureus identification, and for the detection of methicillin resistance. Briefly 2 μL of meCA and nuc primers (10 μmol), 3 μL of 16S rRNA specific primers (10 μmol), 5 μL of dNTP mixture (2.5mM), 5 μL of 10xPCR buffer, 4 μL of MgCl2 (25mM), 0.4 μL of Taq polymerase (TaKaRa, Tokyo, Japan) and 2.5 μL of template DNA were added to PCR mixture and made up to 50 μL by adding distilled water. Amplification conditions consisted of 10 min at 94°C, followed by 23 cycles of 1 min at 94°C, 1 min at 51°C, and 2 min at 72°C, with a final step of 5 min at 72°C. The amplified DNA fragments were evaluated following the gel electrophoresis on a 1.5% agarose (Bio Basic Inc., Totonto, Canada) gel stained with ethidium bromide. Nested PCR followed by Alu restriction enzyme dependent RFLP method was used to determine the polymorphism in coagulase gene regions of S. aureus isolates.[19]. Primers to replicate the coagulase gene region described previously by Goh et al.[18] were used in nested-PCR assays. PCR mixture was prepared by adding 2 μL of each COA1 and COA4 primers (10 μmol), 5 μL of dNTP mixture (2.5mM), 5 μL of 10xPCR buffer, 4 μL of MgCl2 (25mM), 0.4 μL of Taq polymerase (TaKaRa, Tokyo, Japan) and 26.6 μL ultra distilled water to obtain a 50 μL of final mixture, subsequently 5 μL of DNA extract was added and amplified. Fifty μL of similar mixture was prepared for the second cycle of nested PCR but this time 2 μL of COA2 and COA3 primers (10 μmol) were used as primers and 1.5 μL PCR product obtained from previous amplification as target DNA. DNA amplification was performed by pre-denaturation at 95°C for 5 min followed by 40 amplification cycles of 95°C for 30 sec, 55°C for 2 min, 72°C for 4 min and final extension at 72°C for 5 min. Ten μL of nested PCR product was digested by Alu restriction enzyme (TaKaRa, Tokyo, Japan) according to the manufacturer’s recommended protocol. Both PCR
product and restricton digest fragments were detected by electrophoresis through a 3% agarose gel with Φx 174-Hae III Marker (TaKaRa, Tokyo, Japan) and 100 bp marker (Bio Basic Inc., Totonto, Canada).

Note: Ethics committee approval has been taken from AKU HADYEK on 01.04.2010 with the number 81.

**RESULTS**

**Bacteriological Studies Results**

Eighty-three *S. aureus* were isolated from milk samples. According to the results of susceptibility tests, 82 out of 83 isolates (98.8%) were resistant at least one of 16 antimicrobial agents involved in the study. In this experiment 53 isolates (63.8%) were found to be resistant to penicillin; 52 (62.67%) to trimethoprim/sulphamethoxazole; 51 (61.5%) to ampicillin; 40 (48.2%) to erythromycin; 29 (34.9%) to tetracycin; 18 (21.6%) to ciprofloxacin, 16 (19.3%) to clindamycin, 13 (15.6%) to chloramphenicol; 8 (9.6%) to gentamicin; 5 (6.0%) to cefoxitin; 4 (4.9%) to vancomycin; 3 (3.6%) to cephalotin; 2 (2.4%) nafcillin; one (1.2%) to oxacillin and one to (1.2%) furazolidon. No resistance was seen in one (1.2%) to ampicillin, 40 (48.2%) to erythromycin; 29 (34.9%) to tetracyclin; 18 (21.6%) to ciprofloxacin, 16 (19.3%) to clindamycin, 13 (15.6%) to chloramphenicol; 8 (9.6%) to gentamicin; 5 (6.0%) to cefoxitin; 4 (4.9%) to vancomycin; 3 (3.6%) to cephalotin; 2 (2.4%) nafcillin; one (1.2%) to oxacillin and one to (1.2%) furazolidon. No imipenem resistance was seen in the *S. aureus* isolates. There were significant differences between antimicrobial susceptibilities of *S. aureus* isolates ($\chi^2=459.03; P<0.01$).

As a consequence, according to antibiogram results, staphylococci have gained resistance to some commonly used antibiotics. That’s why it is recommended that it should not be used antibiotic without making an antibiogram.

**DISCUSSION**

β-Lactam antibiotics are commonly used in cattle mastitis treatment. Penicillin resistance may be related to national policies about usage of antimicrobial drugs and differences about animal raising systems [8]. The highest penicillin resistance was reported to be in Ireland (71.4%) and England (67.3%) within the European countries followed by 50% in USA. It was rather low in Denmark (18.7%) and in Norway (2%) which were the Scandinavian countries [5,19]. It was also shown by Sori et al.[20] that resistance against penicillin was quite high (87.2%) in South-West Ethiopia. There are studies about cattle mastitis showing that penicillin resistance is high in staphylococcus bacteria from isolates in different regions of Turkey. Guler et al.[2] reported the highest resistance of Staphylococcus bacteria from isolates in different regions [19]. The highest penicillin resistance was reported to be in Ireland (71.4%) and England (67.3%) within the European countries followed by 50% in USA. 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Antimicrobial Susceptibility Profiles...

In Turkey, resistance against trimethoprim/sulphamethoxazole and ampicillin were considerably high. These antimicrobial agents are usually preferred for the treatment of mastitis cases. Coagulase production is an important phenotypic specification to identify *S. aureus*. Gene coagulase is the most important virulence factor for *S. aureus*. It is reported that both counts and localisations of Alu I restriction regions in 3' ends of gene coagulase contain a sequence of 81 base pairs which is different between *S. aureus* isolates [18].

The classification of *S. aureus* isolates depending on gene coa is a simple method for molecular typing and can be assumed as a validation test [18,22]. Aslantas et al. [9] reported that RFLP models of gene coagulase of *S. aureus* isolates from cattle mastitis showed a great diversity. They determined that coagulase gene polymorphism of *S. aureus* isolates from mastitic cow milk by RFLP method using Alu I enzyme produced 9 different genotype strains. Rodrigues da Silva and Silva [22], reported that there were 49 different types of RFLP samples after digestion with Alu I enzyme. Raimundo et al. [23] examined coa gene type of 151 samples of cattle *S. aureus* isolates from 76 farms.

### Table 2. Typing of isolates based on PCR and AluI digestion

<table>
<thead>
<tr>
<th>Number of Isolates (%)</th>
<th>RFLP Type</th>
<th>Sizes of PCR Products (approx. bp)</th>
<th>AluI Profiles*</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 (25.3%)</td>
<td>Type-I</td>
<td>300</td>
<td>+ + +</td>
</tr>
<tr>
<td>51 (61.4%)</td>
<td>Type-II</td>
<td>300</td>
<td>+ - +</td>
</tr>
<tr>
<td>3 (3.6%)</td>
<td>Type-III</td>
<td>300</td>
<td>- - +</td>
</tr>
<tr>
<td>8 (9.6%)</td>
<td>Type-IV</td>
<td>290,870</td>
<td>+ - +</td>
</tr>
</tbody>
</table>

* Results for only fragments of 81 bp or multiples are shown

### Table 3. Distribution of *S. aureus* isolates within dairy farms, antimicrobial sensitivities and RFLP types

<table>
<thead>
<tr>
<th>Origin of the Samples (number of farms)</th>
<th>Antimicrobial Agent</th>
<th>RFPL Type A(4)</th>
<th>RFPL Type B(5)</th>
<th>RFPL Type C(3)</th>
<th>RFPL Type D(4)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RFPL Type I</td>
<td>RFPL Type II</td>
<td>RFPL Type III</td>
<td>RFPL Type IV</td>
<td></td>
</tr>
<tr>
<td>P</td>
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<td>5</td>
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<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>DA</td>
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<td>4</td>
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<td>-</td>
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<td>-</td>
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<tr>
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<tr>
<td>CTX</td>
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</tr>
<tr>
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<td>24</td>
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<td>2</td>
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</tr>
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<td>8</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>17</td>
</tr>
</tbody>
</table>

| RFPL Type I                          | 11                  | -              | -              | -              | -              | 21    |
| RFPL Type II                         | -                   | 44             | -              | -              | -              | 51    |
| RFPL Type III                        | -                   | -              | 3              | -              | -              | 3     |
| RFPL Type IV                         | -                   | -              | 6              | -              | -              | 2     |


and Norway. Besides the penicillin, resistance against trimethoprim/sulphamethoxazole and ampicillin were considerably high. These antimicrobial agents are usually preferred for the treatment of mastitis cases in Turkey. Coagulase production is an important phenotypic specification to identify *S. aureus*. Gene coagulase is the most important virulence factor for *S. aureus*. It is reported that both counts and localisations of AluI restriction regions in 3' ends of gene coagulase contain a sequence of 81 base pairs which is different between *S. aureus* isolates [18]. The classification of *S. aureus* isolates depending on gene coa is a simple method for molecular typing and can be assumed as a validation test [18,22]. Aslantas et al. [9] reported that RFLP models of gene coagulase of *S. aureus* isolates from cattle mastitis showed a great diversity. They determined that coagulase gene polymorphism of *S. aureus* isolates from mastitic cow milk by RFLP method using AluI enzyme produced 9 different genotype strains. Rodrigues da Silva and Silva [22], reported that there were 49 different types of RFLP samples after digestion with AluI enzyme. Raimundo et al. [23] examined coa gene type of 151 samples of cattle *S. aureus* isolates from 76 farms
by using Coa2 and Coa3 primers and found 6 types of PCR. Su et al.[8] investigated coag gene diversity in *S. aureus* isolates from 4 countries. They reported 5 genotypes were dominant for each country. However, dominant types changed according to the geographical regions. Karahan and Cetinkaya [24] reported that 83.9% were produced single band and 16.1% produced 2 bands after coa gene amplification in PCR results of 161 coa positive single band and 16.1% produced 2 bands after coa gene amplification. They found that 23 different types of restriction profiles in RFLP results by using AluI enzyme. Guler et al.[2] investigated 125 *S. aureus* isolates which had antibiotic resistance and found that there were 4 types of coagulase gene. In the present study, 90.4% of the isolates produced single band and 9.6% of them produced double bands after coa gene amplification. In this study, 51 out of 83 *S. aureus* isolates were obtained from 7 dairy farms and each of them produced bands approximately 300 bp in length. After RFLP those isolates produced 2 bands (81, 243 pb). Remaining 32 isolates were obtained from 9 different dairy farms and produced 2 types of PCR products of 300 and 290, 870 bp. It was also found that 24 samples to have 2 different types of 300 bp product after RFLP (81, 162, 243 bp and 243 bp) while PCR products of 290, 870 bp in length were produced 2 bands of 81, 243 bp in length after RFLP. This study showed that there were 4 different types of *S. aureus* as Type I, II, III and IV in Middle Anatolia region upon classification by PCR-RFLP. Besides Type II was the most common one to be present in 61.4% of the isolates. Eventually, 33 out of 51 Type II isolates were found to be resistant to penicillin. 6 out of 8 Type IV isolates were resistant to trimethoprim-sulfamethoxazole and, 13 out of total 21 Type I isolates were resistant to ampicillin amongst antibiotics involved in the trial. All of 83 isolates were sensitive to imipenem. However, only 1 isolate were resistant to nafcillin and furazolidon (Table 3).

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