Investigation of Antibiotic Susceptibility and Presence of Plasmids in Staphylococci Isolated from Cow Milk with Subclinical Mastitis*

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SUMMARY

The purpose of this study was to investigate the susceptibility of Staphylococci isolates from cow milk with subclinical mastitis to some antibiotics and to determine the presence of plasmids in isolates. For this purpose, a total of 51 Staphylococci isolates were examined. These isolates were isolated from 173 milk samples of dairy cows with subclinical mastitis. It was found that 47 (92.15%) isolates were coagulase-negative and 4 (7.84%) were coagulase-positive. These isolates were found to be susceptible to 6 different antibiotics frequently used in mastitis. Three (5.88%) of the 51 Staphylococci isolates were resistant to oxytetracycline, 3 (5.88%) to sulphonmethoxazole-trimethoprim, 6 (11.76%) to novobiocin, 11 (21.56%) to erythromycin, and 24 (47.05%) to penicillin-G. Twenty seven (52.94%) isolates were susceptible to penicillin-G, 40 (78.43%) to erythromycin, 45 (88.23%) to novobiocin, 48 (94.11%) to oxytetracycline, 48 (94.11%) to sulphonmethoxazole-trimethoprim, and 51 (100%) to amoxicillin-clavulanic acid. Plasmids were detected in 16 (31.37%) of the isolates. One (6.25%) of these isolates with plasmid was resistant to erythromycin, 2 (12.50%) to oxytetracycline and 4 (25.00%) to penicillin-G. Twelve (75.00%) of them were found to be susceptible to penicillin-G, 14 (87.50%) to oxytetracycline, 15 (93.75%) to erythromycin, and all of them (100.00%) to novobiocin, amoxicillin-clavulanic acid and sulphonmethoxazole-trimethoprim. In conclusion, although plasmids were found in 16 (31.37%) of the coagulase-negative isolates, there were no plasmids in coagulase-positive isolates.

Key Words

Milk, Staphylococci, Antibiotic resistance, Plasmid, Subclinical mastitis

ÖZET

Çalışmanın amacı, subklinik mastitli inek sütlerinden izole edilen Stafilocokk izolatlarının bazı antibiotiklere duyarılığının araştırılması ve izolatlarda plazmit varlığının belirlenmesidir. Bu amaçla, toplam 51 Stafilocokk izolat incelenmiştir. Izolatlar subklinik mastitli ineklerden alınan 173 süt örneğinden izole edildi. Bu izolatlardan 47 (%92.15)'si koagulaz negatif, 4 (%7.84)'ü ise koagulaz pozitif olarak değerlendirildi. İzolatlar, mastitistide sıkıla kullanılan 6 farklı antibiotikye duyarılı bulunmuş. İzole edilen 51 adet Stafilocokk izolatından 3 (%5.88)'ünün oksitetrasikline, 3 (%5.88)'ünün sulfatmetoksazol-trimetoprim kombinasyonuna, 6 (%11.76)'sının novobiyanın, 11 (%21.56)'sının eritromisinin, 24 (%47.05)'ünün penisilin-G'ye dirençli; 27 (%52.94)'sının penisilin-G'ye, 40 (%78.43)'sının eritromisinin, 45 (%88.23)'sının novobiyanın, 48 (%94.11)'sının oksitetrasikline, 48 (%94.11)'sının sulfatmetoksazol-trimetoprim kombinasyonuna, 51 (%100.00)'sının amoksikilin-klavulanık asit kombinasyonuna duyarılı olduğu tespit edildi. İzolatların 16 (%31.37) tanesinde plazmit tespit edildi. Plazmit taşıyan izolat 1 (%6.25)'iri eritromisine, 2 (%12.50)'sı oksitetrasikline, 4 (%25.00)'u penisilin-G'ye dirençli idi. İzolatların 12 (%23.50)'u penisilin-G'ye, 14 (%27.50)'u sulfatmetoksazol-trimetoprim kombinasyonuna, 51 (%100.00)'u amoksikilin-klavulanık asit ve sulfatmetoksazol-trimetoprim kombinasyonlarına duyarılı bulunmuş. Sonuç olarak koagulaz negatif sütlerin 16 (%31.37)'sında plazmit tespit edilmiş, koagulaz pozitif sütlerde plazmit tespit edilmemiş.

Anahtar Kelimeler Süt, Stafilocokk, Antibiyotik direnci, Plazmit, Subklinik mastitis

INTRODUCTION

Staphylococci isolates are the causative agents of many opportunistic infections in humans and animals. Some species of these organisms are recognized as etiological agents of bovine clinical and subclinical mastitis in the world (Trinidad et al. 1990; Rajala-Schultz et al. 2004; Melchior et al. 2007), including Turkey (Alisarlı and Solmaz 2003; Güler et al. 2005). Staphylococcal mastitis is encountered as infections with usually acute and rarely chronic courses in cows, sheep, and goats. The infection may cause hardening of udders, atrophying them, and in some cases death due to toxemia. The preliminary and constructive factors for appearance of the mastitis problem in cows are examined in three groups, which are the individual characteristics of the cow, the environmental conditions, and microorganisms.
Resistance to antibiotics constitutes one of the most important concerns about health at the beginning of the 21st century. Also, the Staphylococci show resistance to some antibiotics widely (Kono et al.1983; Anderson et al.2006). The pharmaceutical industry has developed oxazolidinone, lipopeptides, injectable streptogramins, ketolides, glycylcyclines, second-generation glycopeptides, and new fluorquinolones against these problems. However, the clinical use of these agents will constitute new selective pressures and continue triggering resistance development (Woodford 2005).

Plasmids are double-stranded autonomous DNA molecules and carry certain specific genetic information. As they convey resistance to antibiotics and heavy metal, toxin formations, pilus production and virulence factors to the bacterium, they bring some advantages to it (Arda 1997; Moroni et al.2006; Melchior et al. 2007).

The purpose of this study was to investigate the susceptibility of Staphylococci isolates isolated from cow milk with subclinical mastitis to some antibiotics and to determine the presence and prevalence of plasmids in these isolates.

MATERIALS and METHODS
Isolation and identification of Staphylococcus spp. from milk samples
In this project, a total of 173 milk samples obtained from dairy cows in central Van and its villages between October 2006 and June 2007. Samples were collected under aseptic conditions by hand-milking in accordance with the recommendations of the International Dairy Federation (IDF) (IDF 1998). The California mastitis test (CMT) was applied to the milk samples. For this purpose, 2 ml of milk from different quarters was collected into each quadrant of a plastic CMT paddle with 4 cups. An equal amount of CMT reagent (2% aril alkaline sulfate, 0.01% bromcresol purple, 15 ml 10% NaOH, and 1000 ml distilled water) was added to it. The mixture in the CMT paddle was rotated for 15-20 sec. The milk samples which became thicker and purple were thought to have mastitis. The samples which were found to have mastitis were put into sterile tubes and were isolated in this study.

Isolation of Staphylococcus spp. was performed by described method. On the other hand, these isolates were harvested in mannitol salt agar (Oxoid, Hampshire, England), oxytetracycline (30 μg, Oxoid), penicillin-G (10 μg, Oxoid), novobiocin (30 μg, Oxoid), erythromycin (5 μg, Oxoid), and sulphamethoxazole-trimethoprim (25 μg, Oxoid) by Bauer et al. (1966). Antibiotics used in the study were preferred for the often recommended in the treatment of mastitis in the Van region.

Plasmid isolation
The method stated by Mansi et al. (1999) was modified for plasmid isolation from Staphylococcus isolates. Briefly, isolated bacteria were inoculated in 5 ml Lauria broth (tryptone 10 gr, yeast extract 5 gr, NaCl 10 gr, distilled water 1000 ml, pH: 7.2) and incubated at 37°C, overnight. After the incubation, 1 ml of the culture was centrifuged in an eppendorf tube at 5000 rpm for 2 min at room temperature (RT). The supernatant was discarded and the pellet was washed twice with TE buffer (10 mM Tris, 1 mM EDTA, pH: 8.0) (solution I). The pellet obtained was then added with 200 μl of freshly prepared solution II (1 M NaOH containing 1% SDS) and then the suspension was mixed. The eppendorf tube was kept in ice for 5 min. It was added with 150 μl of cooled solution III (11.5 ml of glacial acetic acid, 60 ml of 5 M potassium acetate, 28 ml of distilled water). The contents were mixed carefully and then centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant, which contained DNA of the plasmid and some possible RNA, was transferred into a new eppendorf tube which had 20 μl of RNAse in order to make sure even the slightest amount of RNA was removed. After being kept in ice for 10 min, it was added with a mixture of phenol/chloroform/isoamyl alcohol (24:25:1) (Applichem, Germany) in equal amounts to the supernatant. After the contents of the eppendorf tube were mixed completely, it was centrifuged at 12,000 rpm at 4°C for 5 min and then the clear supernatant was transferred to a new eppendorf tube. Then, the supernatant was transferred into a new eppendorf tube and two volumes of ethanol (-20°C) was added and mixed well. The eppendorf tube was kept at 4°C for 60 min. The plasmid DNA was precipitated at 13,000 rpm for 10 min at RT. The supernatant was removed and it was put on paper tissue upside down so that ethanol was removed from the eppendorf tube. Plasmid DNA pellets were then washed with 1 ml of ethanol (70%) twice at 4°C. After the supernatant’s removal, big drops of ethanol were removed. The rest of the ethanol was removed and then plasmid DNA was dissolved in 50 μl of TE buffer and then the DNA was kept at 4°C for processing.

Agarose gel electrophoresis
The plasmid samples were analyzed by electrophoresis on 0.7% (w/v) agarose gel at 80 mV for 2 h and stained with ethidinium bromide (0.5 μg/ml). Ten μl of extracts with suspected plasmids and 3 μl loading buffer were mixed and separated (Mansi et al.1999).
Statistical analysis
The results of antibiotic susceptibility test and plasmid isolation were evaluated in percentage terms (Sümäßlu and Sümbüloğlu, 2002).

RESULTS
Staphylococci isolates
Fifty one Staphylococcus spp. were isolated from 173 milk samples. It was found that all the isolates examined in Gram positive coccius that displayed a cluster-type array, catalase, coagulase, oxidase, O/P (fermentative) tests and mannitol salt agar were positive. Forty seven isolates were found to be coagulase-negative and 4 were coagulase-positive.

Antibiotic susceptibility test
It was found that 3 (5.88%) of the 51 Staphylococci isolates were resistant to oxytetracycline, 3 (5.88%) to sulphamethoxazole-trimethoprim, 6 (11.76%) to novobiocin, 11 (21.56%) to erythromycin, and 24 (47.05%) to penicillin-G. Twenty seven (52.94%) isolates were susceptible to penicillin-G, 40 (78.43%) to erythromycin, 45 (88.23%) to novobiocin, 48 (94.11%) to oxytetracycline, 48 (94.11%) to sulphamethoxazole-trimethoprim, and 51 (100%) to amoxicillin-clavulanic acid (Table 1).

Table 1. Results of antibiotic susceptibility test
<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistant</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>3</td>
<td>5.88</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>6</td>
<td>11.76</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>11</td>
<td>21.57</td>
</tr>
<tr>
<td>Penicillin-G</td>
<td>24</td>
<td>47.05</td>
</tr>
<tr>
<td>Amoxicillin-clavulanic acid</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sulphamethoxazole-trimethoprim</td>
<td>3</td>
<td>5.88</td>
</tr>
</tbody>
</table>

Plasmid detection
It was found that 16 (31.37%) of the 51 Staphylococci isolates were contained plasmids in different masses. All the plasmids were detected in coagulase-negative isolates. Fourteen of the plasmids were of various masses and were resistant to the same antibiotic and was also different, while 2 plasmids were both the same masses (Figures 1 and 2).

Comparison of antibiotic test results and plasmid presence
One isolate (6.25%) that having plasmid was resistant to erythromycin and 2 (12.50%) were resistant to oxytetracycline and 4 (25%) to penicillin-G. Twelve of them (75%) were susceptible to penicillin-G, 14 (87.50%) to oxytetracycline, 15 (93.75%) to erythromycin, and all of them (100%) to novobiocin, amoxicillin-clavulanic acid and sulphamethoxazole-trimethoprim (Table 2).
It was found that of 19 (37.25%) Staphylococci isolates that were susceptible to all of the antibiotics, 9 (17.64%) of them contained plasmids. Eighteen (35.29%) isolates were found to be resistance to any antibiotic and 5 (9.80%) of them contained plasmids. It was also determined that 18 isolates were resistance to 2 different antibiotics and they were contained plasmids (Table 3).

Table 3. Comparative results of antibiotic susceptibility test and the plasmid presence in Staphylococcus isolates

<table>
<thead>
<tr>
<th>Susceptibility to antibiotics*</th>
<th>Number of isolates (n)</th>
<th>Strains containing plasmids</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Strains resistant to only one antibiotic)</td>
<td>19</td>
<td>9</td>
</tr>
<tr>
<td>II (Strains resistant to two antibiotics)</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>III (Strains resistant to more than two antibiotics)</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>IV (Strains resistant to all antibiotics)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
<td>16</td>
</tr>
</tbody>
</table>

*I: Strains resistant to all of the antibiotics, II: Strains resistant to only one antibiotic, III: Strains resistant to two antibiotics, IV: Strains resistant to more than two antibiotics

**Multi-drug resistance isolates**

**DISCUSSION**

Staphylococci are important agents of subclinical and clinical mastitis in dairy cows (Trinidad et al. 1990; Rajala-Schultz et al. 2004; Melchior et al. 2007). In the study of Rajala-Schultz et al. (2004), which was conducted to determine the antimicrobial susceptibility of mastitis pathogens from cow milk with subclinical mastitis, it was stated that 78% of the 202 bacteria that were isolated consisted of coagulase-negative Staphylococci. Costa et al. (2000) reported that 45 Staphylococcus spp. isolates from parenchyma of the udders of dairy cows killed in a slaughterhouse, 33 and 12 were coagulase-negative and coagulase-positive, respectively. In this study, 47 of the 51 Staphylococcus spp. isolated from dairy cows with subclinical mastitis were found to be coagulase-negative, while 4 were coagulase-positive. The percentages of coagulase-negative and positive Staphylococcus spp. were agreement with others (Costa et al. 2000; Rajala-Schultz et al. 2004).

Resistance to antibiotics is the biggest problem encountered in the treatment of bacterial infections (Moroni et al. 2006; Melchior et al. 2007). There are a number of studies related to antibiotic susceptibility of Staphylococci isolated from cases of subclinical and clinical mastitis. (Boyukkara et al. 1991; Costa et al. 2000; Alişarlı and Solmaz 2003). It was found that 3 (5.88%) of the 51 Staphylococcus spp. were resistant to oxytetracycline, 3 (5.88%) to sulphamethoxazole-trimethoprim, 6 (11.76%) to novobiocin, 11 (21.56%) to erythromycin, and 24 (47.05%) to penicillin-G, and 27 of them (52.94%) were susceptible to penicillin-G, 40 (78.43%) to erythromycin, 45 (88.23%) to novobiocin, 48 (94.11%) to oxytetracycline, 48 (94.11%) to sulphamethoxazole-trimethoprim, and 51 (100.0%) to amoxicillin-davulanic acid. According to these results, the two antibiotics to which the greatest resistance were found as penicillin-G (47.05%) and erythromycin (21.56%).

It was observed that the resistance rates of the strains isolated in this study against erythromycin (21.56%) and oxytetracycline (5.88%) were close to the rates of the isolates in Boyukkara et al. (1991), Watts and Salmon (1997), and Alişarlı and Solmaz (2003) against the same antibiotics, and to the rates of the isolates in Moroni et al. (2006) and Güler et al. (2005) against oxytetracycline. Besides, the resistance of the isolates in Boyukkara et al. (1991), Güler et al. (2005), and Anderson et al. (2006) against penicillin-G was higher than that of the isolates in this study against penicillin-G (47.05%).

Kono et al. (1983) reported that the genes of resistance to penicillin and erythromycin could be found on the same plasmid. In our study, however, even though sequence-based genetic examination was not carried out, it was observed that 6 of the strains from which plasmids were detected were resistant to both penicillin and erythromycin, but plasmid masses were all different (Figure 1 and 2).

Although it was stated in Baumgartner et al. (1984) that the strains did not have antibiotic resistance among 85 coagulase-positive Staphylococci isolates isolated from cows with chronic mastitis in 18 farms did not contain plasmids, resistance to antibiotics was also found in strains in which no plasmids were detected in our study. These results indicate that antibiotic resistance may not be related to the presence of plasmids.
Arslan et al. (2004) examined the antibiotic susceptibility of 40 coagulase-positive and 10 coagulase-negative Staphylococcus spp. isolated from cows with mastitis using standard bacteriological methods and their plasmid profiles. It was stated that 38 (95.0%) of 40 coagulase-positive and 9 (90.0%) of 10 coagulase-negative Staphylococci isolates were contained plasmids. However, in our study, while plasmids were detected in 16 of 47 coagulase-negative Staphylococci isolates and none of the 4 coagulase-positive Staphylococci isolates were contained plasmids.

Piccinini and Zecconi (2001) reported that one or more than one plasmids in 9 different masses in 3 of 4 coagulase-positive Staphylococci isolates were detected. However, no plasmids were detected in coagulase-positive Staphylococci isolates isolated in our research. On the other hand, 14 different masses of plasmids were detected in 16 (31.37%) of the coagulase-negative isolates (Figure 1 and 2).

Plasmids were detected in 16 (31.37%) of the Staphylococci isolates in this study. Fourteen of the plasmids were of various masses and the masses of the plasmids detected in different strains that were resistant to the same antibiotics were also different, while 2 plasmids were both the same mass (Figure 1 and 2). All of the plasmids were detected in coagulase-negative strains. Seven of the strains containing plasmids (43.75%) were resistant to antibiotics.

In conclusion, high resistance seen in animals with subclinical mastitis against certain antibiotics clearly indicates that the effectiveness of antibiotic treatment administered without carrying out antibiotic tests will be low. The fact that 16 (31.37%) strains with plasmids were found in 51 Staphylococci isolates show that plasmids have considerable importance in resistance transfer; however, the fact that resistance to various antibiotics was observed in strains with no plasmids detected show that antibiotic resistance is not only dependent on the presence of plasmids.

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