Full Length Research Paper

Detection of the antibiotic resistance genes in *Staphylococcus aureus* isolated from human infections and bovine mastitis

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The present study was carried out in an attempt to detect the distribution of antibiotic-resistant genes of *Staphylococcus aureus* isolates from human infections and bovine mastitis. *mecA*, *msrA*, *msrB*, *aacA-D*, *tetK* and *tetM* genes were selected in order to detect the distribution of antibiotic-resistant genes by multiplex PCR technique. According to the biochemical analysis and detection of 16S-rDNA by PCR method, 108 isolates of 300 human infections samples and 18 strains from 150 bovine mastitis milks were recognized as *S. aureus*. Distributions of antibiotic-resistant genes in human isolates were as follows: (85.18%) *mecA*, (46.29%) *msrA*, (49.07%) *msrB*, (33.33%) *aacA-D*, (80.50%) *tetK* and (66.66%) *tetM* and in bovine mastitis, isolates were seen to be ranging from: (22.22%) *mecA*, (66.66%) *msrA*, (77.77%) *msrB*, (33.33%) *aacA-D*, (55.55%) *tetK* and (50.00%) *tetM*, respectively. Results indicated that all *S. aureus* strains have one or more of the antibiotic-resistant genes. Also, multiplex PCR technique is a fast, practical and appropriate technique for determining antibiotic-resistant genes. Hence, it was possible that the treatment and the right antibiotics were used.

**Key words:** Antibiotic-resistant genes, bovine mastitis, human infections, multiplex PCR, *Staphylococcus aureus*.

INTRODUCTION

*Staphylococcus aureus* is a Gram positive coccus (Steinberg et al., 1996) with circular chromosome located on those pathogenesis and antibiotic-resistant genes (Novick, 1990). It is one of the most important bacteria in Micrococccaceae family, which is also responsible for a wide variety of community- and hospital-acquired infections (Hiramatsu, 1998; Lowy, 1998; Abed El-Jalil et al., 2008). This bacterium causes the pyogenic infections and toxigenic illness in humans and animals (Jahoda et al., 2007). *S. aureus* is one of the major causes of mastitis in cow, which is resistant against multiple antimicrobial drugs (Wang et al., 2008). Different strains of *S. aureus* and some of the coagulase negative staphylococci (CNS) species are agents of community- and hospital-acquired infections (Naimi et al., 2001). Moreover, *S. aureus* is the leading cause of bacterial infections involving the bloodstream, lower respiratory tract, skin and soft tissue in many developed countries (Herold et al., 1998; Gorak et al., 1999).

Inappropriate and the extra-usage of antibiotics have some roles in causing resistant *S. aureus* (Chambers, 1997; Hiramatsu, 1998). In 1940, some *S. aureus* strains showed resistance to penicillin. A decade later, multiple-resistant strains to tetracycline, chloramphenicol and erythromycin were reported (Kirby, 1944; Barber and Rozwadowska-Dowzenko, 1948). Methicillin-resistant *S. aureus* (MRSA) identified in 1960 was initially considered as a nosocomial pathogen (Vandenesch et al., 2006). *S. aureus* is also one of the major causes of mastitis in cow.

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However, mastitis treatment is not usually successful because this disease causes main damages in breast and drugs which are not able to make useful effects in all infection levels (Nickerson, 1993). On the other hand, this bacterium prevents phagocytosis and indirect cell-immunity (Yilmaz et al., 2007) and it also produces an enzyme which prevent most of the penicillin treatments (De Oliveira et al., 2000). It was also proven that beside methicillin antibiotic, S. aureus is resistant to other antibiotics like beta-lactam, amino-glycosides, fluoroquinolones, lincosamides, macrolides and streptogramins (Chambers, 1987, 1997 Ramdani-Bouguesa et al., 2006), so it does not usually react to antibiotics treatment (Zecconi and Piccinini, 2002; Ochoa-Zarzosa et al., 2008; Wang et al., 2008; Kumar et al., 2010).

S. aureus strains diversify in antibiotic resistance by various mechanisms, such as modification of the ribosomal target site, enzymatic inactivation of the drug, metabolic pathway alteration, efflux pumps and enzyme-elicited cleavage of antibiotics (Schreckenberger et al., 2004; Yilmaz et al., 2007; Ochoa-Zarzosa et al., 2008; Wang et al., 2008; Kumar et al., 2010). The antibiotic-resistant genes: mecA (methicillin), aacA-D (aminoglycosides), tetK, tetM (tetracyclines), ermA, ermB, ermC (macrolide–lincosamide–streptogramin B), msrA (macrolides) and linA (lincosamides) have been reported in last decade among the isolates of S. aureus (Ochoa-Zarzosa et al., 2008; Wang et al., 2008). mecA gene encodes PBP2a, aacA-D gene encodes bi-functional enzyme (Kumar et al., 2010), msr gene in staphylococci has caused resistance to macrolides and streptogramin B that is named as MSB phenotype and affects on efflux pumps activity (Merino-Díaz et al., 2007; Kumar et al., 2010). erm(A,C) gene causes modification of the bacteria’s ribosome target site and encodes enzymes that by methylation of 23S rRNA causes reduction in connection of MLSβ antibiotic group to ribosome (Drinkovic et al., 2001; Mlynarczyk et al., 2007; Kumar et al., 2010), and tetK, tetM for modification of the ribosome or effluxing (Kumar et al., 2010). Therefore, the detection of antibiotic-resistant genes in S. aureus strains with high virulent requires an effective guideline for the use of antibiotics (Ochoa-Zarzosa et al., 2008; Wang et al., 2008; Kumar et al., 2010).

Due to the shortage of information of antibiotic-resistant in S. aureus in Iran, this study was carried out in an attempt to detect antibiotic-resistant genes such as those resistant genes to methicillin (mecA), macrolides (msrA, msrB), aminoglycosides (aacA-D) and tetracyclines (tetK, tetM) by the use of multiplex PCR method in S. aureus isolated from human infections and bovine mastitis.

MATERIALS AND METHODS

Detection of S. aureus

A total of 126 strains of S. aureus from various infections in human and bovine mastitis were examined. The samples taken from clinical materials (150 milk samples from cows and 300 samples of wound infections, urine infections, coetaneous absesses and obstetric infections from human) were directly cultured onto 7% sheep blood agar and incubated aerobically at 37°C for 48 h. After incubation, suspicious colonies were examined by the use of morphologies compatible with Staphylococcus spp. and were transferred to Tryptic Soy Broth (TSB) (Merk) and Tryptic Soy Agar (TSA) (Merk). After growth, staphylococci were identified on the basis of colony characteristics, Gram staining, pigment production, hemolytic and the following biochemical reactions: catalyses activity, coagulated test (rabbit plasma), Oxidase test, O/F test with glucose, resistance to bacitracin (0.04 U), mannitol fermentation on Chapman agar, urease, nitrate reduction, novobiocin resistance, phosphatase, deoxyribonuclease (DNase) test, carbohydrate (xylose, sucrose, trehalose and maltose, fructose, lactose, mannose) fermentation tests (Zmantar et al., 2008; Kumar et al., 2010).

Amplification of antibiotic-resistant genes by multiplex PCR technique

The detection of antibiotic resistance genes by PCR was done using forward and reverse primers previously introduced by Kumar et al. (2010). PCR primers were chosen from the antibiotic resistance genes mecA, aacA-D, tet K, tet M, msrA and msrB and 16s-rDNA as listed in Table 1. S. aureus strains were grown on sheep blood agar plates overnight at 37°C. One colony was suspended in 1 ml LB broth for 24 h at 37°C. Chromosomai mal DNA was extracted by DNA Genomic Purification Kit (Fermentas, Germany). PCR was performed in a PCR thermocycler (Eppendorf Mastercycler, Eppendorf-Nethel-Hinz GmbH, Hamburg, and Germany).

The presence of the mecA, aacA-D, tet K, tet M, msrA and msrB genes encoding methicillin, aminoglycosides, tetracyclines and macrolides resistance was examined in 126 strains using multiplex PCR (Zmantar et al., 2008). Multiplex PCR assays were performed in 25 µL PCR mixtures 1 and 2. The mixture 1 contained 1 U of Taq DNA polymerase (Fermentas, Germany), 2.5 µL PCR buffer (10×), 1 µM each forward and reverse primers of mecA, tet K and tet M gene, 150 µmol/L of each dNTP and DNA template (50 ng). Using thermal cycling, the target genes were amplified (94°C 5 min, 30 cycles of 1 min at 95°C for the denaturation step and 1 min at 55°C for the annealing-extension step and 90 s at 72°C for the extension step). In mixture 2, the forward and reverse primers of the genes aacA-D, msrA and msrB (2.5 µM) were used. Ten microliters of PCR product was resolved on a 2% agarose gel containing 0.5 mg/ml of ethidium bromide in Tris–borate–EDTA buffer at 80 V for 1 h.

Statistical analysis

The data were analyzed using SPSS ver. 16.0 statistical software and a Chi-square test analysis was performed. Also, differences were considered significant at values of p<0.05.

RESULTS

Based on biochemical characteristics, from 450 tested samples, 126 isolates were identified as S. aureus. Out of 126 isolates, 108 had human and 18 strains were reported to have bovine origin. Amplification of 16s-rDNA confirmed all the 126 staphylococcal isolates as
Table 1. Oligonucleotide primers for amplification of antibiotic resistance genes in Staphylococcus aureus.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Size of Product (bp)</th>
</tr>
</thead>
</table>
| mecA   | F : AAAATCGATGGTAAAGGTTGCG  
        | R : AGTTCTGCACTAGCCGTTCGGGC         | 532                  |
| aacA-D | F : TAATCCAAGGAATAAGGCGAAGGC  
        | R : GCCACACTATCAATAACCCTACT        | 227                  |
| tet K  | F : GTAGCGACAATAGGTAATAGT  
        | R : GTAGTGACAATAAACCTCTCA          | 360                  |
| tet M  | F : AGTGGAGGCAATTACAGAA  
        | R : CATATGTCTCGAAGCTCTCA           | 158                  |
| msrA   | F : GGCAAAATGGTAAAGGTTGCG  
        | R : AGTTATACATGAAAAGTGCAGTCTCTGA   | 940                  |
| msrB   | F : TATGATATCCATAATATTACATGACAGTTGCTCTGA  
        | R : AGTTATACATGAAAAGTGCAGTCTCTGA   | 595                  |
| 16s-rDNA | F : GTAGGGCCAGTTACC  
         | R : CCGACATCAAGCGTCAG              | 228                  |

Table 2. Distribution of antibiotic-resistant genes in Staphylococcus aureus isolated of human infections and bovine mastitis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>mecA(%)</th>
<th>msrA(%)</th>
<th>msrB(%)</th>
<th>aacA-D(%)</th>
<th>tetK(%)</th>
<th>tetM(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (n=108)</td>
<td>92 (85.18)</td>
<td>50 (46.29)</td>
<td>53 (49.07)</td>
<td>36 (33.33)</td>
<td>87 (80.50)</td>
<td>72 (66.66)</td>
</tr>
<tr>
<td>Bovine (n=18)</td>
<td>4 (22.22)</td>
<td>12 (66.66)</td>
<td>14 (77.77)</td>
<td>6 (33.33)</td>
<td>10 (55.55)</td>
<td>9 (50.00)</td>
</tr>
<tr>
<td>Total (n=126)</td>
<td>96 (76.19)</td>
<td>62 (49.20)</td>
<td>67 (53.17)</td>
<td>42 (33.33)</td>
<td>97 (76.98)</td>
<td>81 (64.28)</td>
</tr>
</tbody>
</table>

S. aureus. The distribution of msrB in human population was 49.07% and in bovine population was 77.77%. On the other hand, the prevalence of msrA was considerably lower than the msrB. Only 50 isolates of human and 12 isolates of bovine origin yielded the band for msrA.

Among methicillin-resistant isolates, 96 (92 isolates of human and 4 isolates of bovine population) could amplify the mecA gene. Moreover, 33.33% of isolates were positive for aacA-D. The occurrence of tetK (80.50% in human and 55.55% in bovine isolates) among the bacterial isolates was significantly higher than tetM (66.66% in human and 50.00% in bovine population) gene. The details of distribution of antibiotic-resistant genes among the isolates are presented in Table 2. Besides, Pearson’s Chi-square test revealed an association that was significant (P<0.05) between mecA and msrB in bovine isolates and mecA and aacA-D in human isolates. Moreover, three strains (16.66%) were resistant to single antibiotic, while 5 strains (27.77%) showed resistance to 2 antimicrobial agents. Multi-resistance which is defined as resistance to 3 or more of drug tested was found in 55.55% of S. aureus strains isolated from bovine. In human isolates, 16 strains (14.81%) were resistant to single antibiotic and 22 strains (20.37%) showed resistance to 2 antimicrobial agents. Multi-resistance which is defined as resistance to 3 or more of drug tested was therefore found in 70 (64.81%) of S. aureus strains.

DISCUSSION

The present investigation was carried out to investigate the distribution of antibiotic-resistant genes including, mecA, msrA, msrB, aacA-D, tetK and tetM by multiplex PCR technique in S. aureus strains isolated from human infections and cow’s milk. Multiplex PCR assays were successfully developed for the detection of six different resistance genes of genomic DNA of S. aureus isolates from human and bovine clinical samples. The results indicated that the prevalence of resistance to tetracycline was 76.98 and 64.28% for tetK and tetM genes, and to
methicillin and aminoglycosides were 76.19 and 33.3%, respectively. Also, the prevalence of gene resistance for macrolides was 49.2 and 53.17% for msrA and msrB genes, respectively (Table 2).

The research by Vandensch et al. (2003) that was carried out on 117 CA-MRSA isolates from the United States, France, New Zealand and Western Samoa by PCR for 24 virulence factors, recognized resistant to the methicillin. In a survey by Turutoglu et al. (2006) on bovine mastitis, the antibiotic susceptibility test was carried out on 103 S. aureus and 136 coagulase-negative staphylococcus (CNS) strains, only 35 (10 S. aureus and 25 CNS) of the isolates were susceptible to all antibiotics being tested, while the remaining 204 isolates were resistant at least to one of the antibiotics. Among being tested, while the remaining 204 isolates were resistant at least to one of the antibiotics. Among Staphylococci, 18 S. aureus strains and 31 CNS isolates were found phenol-typically resistant to penicillin G, ampicillin, amoxicillin and cloxacillin. Of 68 S. aureus isolates, 38 (55.9%) were resistant to erythromycin, clindamycin and gentamicin. Different antibiotic-resistant genes combinations were presented. All isolates lacked amplification of vatA, vatB, ermA and ermC genes, which showed significant prevalence of resistant to multiple antibiotics. The results of the present investigation, just as other researches done in the different parts of the world, are representatives of significant of S. aureus strains that are resistant to the antibiotic in level of animal and human populations, which could arise due to unlimited consuming of antibiotic in the society and animal treating centers.

Multiple PCR technique is a very fast and inexpensive technique for detecting antibiotic resistant genes in resistant strains. It is required to control the transfer or spread of pathogenic strains and from separate cows having mastitis in order to prevent the transfer of infection from one cow to another. Furthermore, antibiotic susceptibility tests should be done besides detecting bacterial factors in order to enhance treatments for decreasing infections of human staphylococci and bovine mastitis.

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