Staphylococcus aureus is a Gram-positive facultative anaerobic bacterium that is both catalase and coagulase positive\(^1\). S. aureus is the most dangerous of staphylococcal bacterial infections that causes a variety of diseases in animals and humans.

Foodborne diseases, commonly called food poisoning, are an important public health and hygiene in the world and S. aureus is the most reported cause of foodborne intoxications.

Detection of Antibiotic Resistance Genes in Staphylococcus aureus Strains Isolated from Cow’s Milk using Multiplex PCR Assay

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Mastitis is an important disease in dairy herds, typically caused by bacterial infection such as Staphylococcus aureus. Antibiotic resistance in S. aureus is worldwide public health problem and considerable concern that continues to grow. The objective of the present study was to investigate the antibiotic susceptibility of S. aureus strains by disk diffusion test and determination of meca, ermA, ermB, and ermC genes, respectively. Multiplex PCR assay showed that 35 (41.18%), 25 (29.41%), 20 (23.53%), 17 (20%), and 16 (18.82%) of S. aureus samples, contained meca, ermA, ermB, and ermC genes, respectively. Furthermore, the data may be helpful for prescription of best drugs for control of the infection caused by this bacterium in cows for the reduction of mastitis.

Key words: Staphylococcus aureus, Antibiotic resistance, Cows Milk, Multiplex PCR.
Furthermore, *S. aureus* is the most common etiological organism responsible for 30-40% of mastitis in dairy herds and its resistance against multiple antimicrobials has been described in several organisms. For example, enzymes (EreA and EreB) that hydrolyse the lactone ring of the macrocyclic nucleus and phosphotransferases that inactivate macrolides have been reported in *S. aureus*\(^{16, 17}\). The purpose of present study was to determine the antibiotic resistance genes (*mecA*, *ermA*, *ermB*, *ermC*, and *msrA*) in *Staphylococcus aureus* strains isolated from cow’s milk using multiplex PCR technique and disk diffusion test in southwest Iran.

**MATERIALS AND METHODS**

**Collection of samples**

In the present study, 100 cow’s milk samples that suspected for clinical mastitis were collected from 8 cattle industries and 5 traditional centers in Chaharmahal Va Bakhtiari province located in Southwest Iran.

**Staphylococcal isolates**

The samples were cultivated in sheep blood agar (SBA) (Merck, Darmstadt, Germany) and incubated in 37°C for 24 hours. The black, grey or white colonies of *Staphylococcus* were cultivated onto blood agar plates containing 5% sheep blood and identified using the catalase, oxidase, and the coagulase tests. The isolates were stored in tryptose soy broth (TSB; Oxoid, Basingstoke, UK) supplemented with 20% glycerol at 80°C until studied.

**Disk diffusion test**

All *S. aureus* isolates were investigated for their antibiotic resistance or susceptibility by disk diffusion test (D-zone test) on SBA using the following antibiotics: clindamycin (Cc), erythromycin, methicillin (Met), and oxacillin (Ox). Antibiotic disks were applied by a dispenser within 15 min after inoculation. Inhibition zone diameters were measured after 16-18 h of incubation at 37°C, but 24 h for Met.

**Minimal inhibitory concentrations (MICs)**

The MICs were determined using a standardized microdilution test (Veterinary plate for staphylococci, Trios, Prague, Czech Republic). The MICs interpretative criteria were based on the recommendations given in document M100-S16 of the Clinical and Laboratory Standards Institute. *S. aureus* ATCC 25923 served as a reference strain for quality control purposes.
DNA isolation

Black, grey or white colonies of S. aureus that cultivated onto SBA were selected randomly for investigation of antibiotic resistance genes. Then, bacterial DNA was extracted from each colonies using DNP™ Kit (CinnaGen, Iran), according to the manufacturer’s protocol. The isolated DNA was quantified by spectrophotometric measurement at a wavelength of 260 nm according to the method described by Sambrook and Russell. The extracted DNA of each sample was kept frozen at -20°C until used.

Amplification of antibiotic resistance genes

In present study multiplex PCR technique were used for investigation of antibiotic resistance genes of S. aureus strains isolated from cow’s milk. The oligonucleotide primers described by Martineau et al., were used in this study. The sequences of primers for amplification of mecA, ermA, ermB, ermC, and msrA genes of S. aureus strains are given in Table 1.

S. aureus ATCC 25923 DNA was used as a positive control. A negative-DNA control was performed by adding 1 µL of sterile ultrapure deionized water. For investigation of antibiotic resistance genes of S. aureus the specimens were amplified in a Gradient Palm Cycler (Corbett Research, Australia) and multiplex PCR reaction was performed in a total volume of 25 µL in 0.5 ml tubes containing 1 µg of genomic DNA, 1 µM of each primers, 2 mM MgCl₂, 200 µM dNTP, 2.5 µL of 10X PCR buffer and 1 unit of Taq DNA polymerase (Roche applied science, Germany). PCR cycles consisted of an initial denaturation step (95°C for 5 min) followed by 30 amplification cycles (denaturation at 94°C for 1 min, annealing at 58°C, and elongation at 72°C for 1 min) with a final elongation at 72°C for 5 min.

Analysis of multiplex PCR products

The amplified products were detected in 1% agarose gel electrophoresis. The electrode buffer was TBE (Tris-base 10.8 g 89 mM, Boric acid 5.5 g 2 mM, EDTA (pH 8.0) 4 ml of 0.5 M EDTA (pH 8.0), combine all components in sufficient H₂O and stir to dissolve). Aliquots of 10 µL of PCR products were applied to the gel. Constant voltage of 80 V for 30 min was used for products separation. The DNA fragment size was compared with a standard molecular weight (100 bp DNA ladder of Fermentas, Germany). After electrophoresis, the amplicons were visualized with ultraviolet light after ethidium bromide (5 µg.mL⁻¹) staining and photographed were obtained in UVIdoc gel documentation systems (UK).

Statistical analysis

Analysis of data and investigation of antibiotic resistance genes of S. aureus were performed using the SPSS version 17.0 computer software (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Cultivation of 100 cow’s milk samples in sheep blood agar and investigation of catalase, oxidase, and the coagulase tests showed 85 specimens (85%) were S. aureus that related to mastitis. The results of present study showed multiplex PCR assay correlated very well with disk diffusion test and MIC determination. There was no discordance between conventional susceptibility testing and PCR for isolated S. aureus strains (Table 2). Electrophoresis of PCR products for detection of S. aureus antibiotic resistance genes revealed 163, 174, 139, 224, and 190 bp(s) for msrA, mecA, ermA, ermB, and ermC, genes, respectively (Fig. 1). Multiplex PCR assay showed that from 85 positive samples for S. aureus 35 (41.18%), 25 (29.41%), 20 (23.53%), 17 (20%), and 16 (18.82%) specimens, contained msrA, mecA, ermA, ermB, and ermC, genes, respectively (Table 3). The results showed that msrA gene (41.18%) is more frequent
Table 1. Primers used for detection of antibiotic resistance genes in *S. aureus* strains isolated from cow’s milk

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number (GenBank)</th>
<th>Oligonucleotide primers sequences</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>msrA</td>
<td>X52085</td>
<td>msrA-F: 5´-TCCAATCATAGCACAAATC-3´ 163</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>msrA-R: 5´-AATACCCTCTATTTTGGTGTG-3´</td>
<td></td>
</tr>
<tr>
<td>mecA</td>
<td>X52594</td>
<td>mecA-F: 5´-AACAGGTGAATTTACATCTGAAG-3´ 174</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mecA-R: 5´-ATTGCTGTTAATATTTTTTGAGTTGAA-3´</td>
<td></td>
</tr>
<tr>
<td>ermA</td>
<td>K02987</td>
<td>ermA-F: 5´-TATCTTTACGTTAGAAGGGAT-3´ 139</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ermA-R: 5´-CTACACTGGCTTAGAGATGAA-3´</td>
<td></td>
</tr>
<tr>
<td>ermB</td>
<td>FN677479</td>
<td>ermB-F: 5´-CTTACCTTGGATATTCACCG-3´ 224</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ermB-R: 5´-GTAAACAGTTGACGATATTCTCG-3´</td>
<td></td>
</tr>
<tr>
<td>ermC</td>
<td>M17990</td>
<td>ermC-F: 5´-TTTACCTGCAACCGTATTGC-3´ 190</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ermC-R: 5´-ATCTTTTagAAAAACCCGTATTC-3´</td>
<td></td>
</tr>
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</table>

Table 2. Correlation between antibiotic susceptibility testing and PCR for investigation of *S. aureus* strains antibiotic resistance genes

<table>
<thead>
<tr>
<th>Resistance genes (antibiotic used for susceptibility testing)</th>
<th>msrA methicillin</th>
<th>mecA oxacillin</th>
<th>ermA clindamycin</th>
<th>ermB erythromycin</th>
<th>ermC erythromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptibility test results</td>
<td>PCR+</td>
<td>PCR-</td>
<td>PCR+</td>
<td>PCR-</td>
<td>PCR+</td>
</tr>
<tr>
<td>Resistant</td>
<td>35</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Susceptible</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>60</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3. Frequency of antibiotic resistance genes of *S. aureus* isolated from cow’s milk samples using multiplex PCR assay

<table>
<thead>
<tr>
<th>Gene</th>
<th>Frequency N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>msrA</td>
<td>35 (41.18)</td>
</tr>
<tr>
<td>mecA</td>
<td>25 (29.41)</td>
</tr>
<tr>
<td>ermA</td>
<td>20 (23.53)</td>
</tr>
<tr>
<td>ermB</td>
<td>17 (20)</td>
</tr>
<tr>
<td>ermC</td>
<td>16 (18.82)</td>
</tr>
</tbody>
</table>

than other antibiotic resistance genes of *S. aureus* isolated from cow’s milk specimens.

*S. aureus* is non-motile, non-spore-forming, and catalase-positive bacteria. *S. aureus* expresses a variety of virulence factors. This pathogen is responsible for causing a wide array of diseases ranging from mild skin infections such as folliculitis and carbuncles to life-threatening conditions such as bacteremia, pneumonia, and endocarditis in human and animals. The bacterium continues to demonstrate the ability to develop resistance to include a broad array of antimicrobial classes, and *S. aureus* is a prominent pathogen in both hospital and the community settings. *S. aureus* strains resistant to methicillin and many other antibiotics are major causes of mastitis and cow disease. Resistance to methicillin is determined by the *mecA* gene, which encodes the low-affinity penicillin-binding protein PBP 2A. MRSA (methicillin resistant *S. aureus*) is present in the nose and on the skin and is shed into the environment by infected or colonized people and animals, indicating that airborne transmission is a possible route for infection.

There is no vaccine available, and the role of passive immunoprophylaxis is unclear. The population at risk increases with more elderly people and more patients receiving immunosuppression or having indwelling catheters and other foreign materials. The numbers of resistant bacteria, MRSA are rising. Within a year after the introduction of semi-synthetic penicillins such as methicillin, there were reports of resistant isolates in 1961. There are many genes role in

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antibiotics resistance of *S. aureus*. In present study we investigated the antibiotic susceptibility by disk diffusion test and determined the *mecA*, *ermA*, *ermB*, *ermC*, and *msrA* antibiotic resistance genes of *S. aureus* strains isolated from cow’s milk using multiplex PCR assay. Multiplex PCR technique showed that 35 (41.18%), 25 (29.41%), 20 (23.53%), 17 (20%), and 16 (18.82%) samples, contained *msrA*, *mecA*, *ermA*, *ermB*, and *ermC*, antibiotics resistance genes of *S. aureus*, respectively. The disk diffusion test confirmed the results obtained from multiplex PCR method. The results of our study showed that *msrA* gene is more frequent than other antibiotic resistance genes of *S. aureus* isolated from cow’s milk specimens and it could be related to increasing of macrolides, lincosamides, and streptogramins (MLS) antibiotics resistant.

There are many studies performed for detection of antibiotic resistance genes of *S. aureus* in human and animals. The frequency of antibiotic resistance genes of *S. aureus* is different in Iran and other parts of world. Zamani et al., (2007) showed that out of a total of 70 *S. aureus* isolates obtained from the patients who consulted with the clinical centers of Hamedan Medical Science University and private laboratory in Iran 50% of the strains (35 cases) in PCR method and 31.4% (22 cases) in antibiotic resistance patterns with disc agar diffusion method were resistance to methicillin\(^5\). Heo et al., (2008) in Korea showed that resistance to oxacillin and methicillin in *S. aureus* isolated from domestic and imported raw meat by the disk diffusion test and minimal inhibition concentration methods, but *mecA* gene not observed\(^6\).

Evaluation of methicillin resistance *S. aureus* isolated from patients in Golestan province-north of Iran showed 67(36.2%) strains were MRSA, which demonstrated 100% resistance to penicillin, ampicillin and COAmoxyclov and 80, 96.2 and 75% resistance to cephotaxime, nalidixic acid and erythromycin, respectively\(^7\).

The study of Mirzaei et al., in 2011 on prevalence of methicillin-resistant *S. aureus* in raw milk and dairy products in Sarab by culture and PCR techniques showed that presence of coagulase positive *S. aureus* and MRSA have become remarkably widespread in dairy product samples\(^8\). They are no detected of *mecA* gene in raw milk and traditional butter isolates while in present results we detected *mecA* gene *S. aureus* isolated from cow’s milk samples.

An antibiotic resistance strain of *S. aureus* is the major groups of bovine mastitis pathogens and worldwide public health problem. In current study *msrA* gene is more frequent than other antibiotic resistance genes and it could be related to increasing of MLS antibiotics resistant. In conclusion, investigation of antibiotic resistance genes of pathogen bacteria such as *S. aureus* is important for prevention and reduces of MRSA and other resistant strains. The results of present study generated a lot of useful information for public health, foodstuff, and dairy industry to control and decrease transmit of *S. aureus* antibiotic resistance strains to human. Furthermore, these data could be helpful for prescription of best drugs for control of *S. aureus* infection in cows.

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