INVESTIGATING OF ENTEROTOXIN GENES BY MULTIPLEX PCR IN STAPHYLOCOCCUS AUREUS STRAINS ISOLATED FROM BOVINE MASTITIS

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ABSTRACT
A total of 40 Staphylococcus aureus strains isolated from bovine mastitis, were analyzed to investigate the presence of the enterotoxin genes sea, seb, seg, seh, sei and sej using Multiplex PCR. 40% of the isolates were positive for one or more toxin genes. The seg gene was found most frequently (32.5%), followed by sej (22.5%), seh (20%), seb (10%), sei (10%) and finally, sea (7.5%). Our results revealed that Multiplex PCR method is simple, sensitive, low cost, relatively rapid and very specific; in addition, it proved to be able to identify several genes that encode toxin at the same time. The results of this study also showed that S. aureus causing mastitis in cows can harbor enterotoxin-encoding genes with seg as the most frequent gene observed amongst the investigated isolates. These findings are important for surveillance purposes, since enterotoxin G should be investigated in human staphylococcal food poisoning outbreaks caused by the consumption of cow milk and dairy products.

Keywords: Staphylococcus Aureus, Enterotoxin Genes, Bovine Mastitis, Multiplex PCR

INTRODUCTION
Staphylococcus aureus is one of the major bacterial pathogens which cause a variety of infectious in humans and animals. This organism plays an important role in the etiology of infectious bovine mastitis (Atashpaz et al., 2010).

Bovine mastitis is a multifactorial disease, which results in the reduction of milk yield, changes in milk composition and increase in the amount of discarded milk. It imposes serious economic losses for the farmers and the dairy industry (Santos et al., 2008).

Some strains of Staphylococcus aureus are pathogenic and responsible for food poisoning by producing enterotoxins (Pereira et al., 2009).

Staphylococcal Enterotoxins (SEs) are a group of single-chain, low-molecular weight proteins (MW, 26900–29600Da), that are very resistant to heat and gastrointestinal proteases such as pepsin, that justifies why they remains active after thermal processing of food and ingestion (El-Huneidi et al., 2006; Fooladi et al., 2010; Pelisser et al., 2009; Rall et al., 2008). SEs are usually divided into the classic (SEA to SEE) and newly described (SEG to SER and SEU) enterotoxins (El-Huneidi et al., 2006).

Several studies have reported the production of SEs or the presence of toxin genes in Staphylococcus aureus from milk and derivates associated with mastitis cows in different countries (De Freitas et al., 2008; Karahan et al., 2009; Omoe et al., 2002; Vimercati et al., 2006).

The genes responsible for encoding SEs are often embedded in mobile genetic elements, such as transposons, prophages, plasmids and pathogenicity islands (Fusco et al., 2011), which may be transferred horizontally between staphylococcal strains. So, enterotoxin genes may play an important role in the evolution of S. aureus as a pathogen (Zhang et al., 2013).

For the above-mentioned reasons, the evaluation of enterotoxin genes in S. aureus isolates can be useful for epidemiological tracing and evolutionary analyses.

There are various methods for the detection of enterotoxigenic bacteria, such as latex agglutination, ELISA, immunodiffusion and RIA. But these methods are not reliable because specific circumstances are necessary for enterotoxin gene expression. However, despite the presence of entrotoxin genes, in specific
circumstances, S. aureus may not have the ability to produce toxin which would lead to negative results (Saadati et al., 2011). In addition, molecular techniques such as PCR and Multiplex PCR are recommended for the detection of S. aureus enterotoxin genes (Fooladi et al., 2010). These techniques are rapid, sensitive, specific, and reliable compared to immunological toxin production assays (Pinto et al., 2005). Another advantage of molecular methods is that strains producing low levels of enterotoxin could be identified by these methods (Saadati et al., 2011). Therefore, they constitute very valuable tools for routine applications (Pinto et al., 2005).

In this work, Multiplex PCR method was used to explore the presence of enterotoxin genes sea, seb, seg, seh, sei, and sej in S. aureus isolates obtained from milk samples of cows with mastitis in Ardabil, a Northwest provincial city in Iran.

MATERIALS AND METHODS

Bacterial Strains

This study used 40 S. aureus strains isolated from milk samples of cows with mastitis that were collected and identified by biochemical tests in a previous study. Isolates were sub cultured on nutrient agar (NA) and identified by biochemical tests, which included catalase, coagulase, mannitol fermentation, and hemolysin, DNase, lecithinase, protease, and lipase production (Chapaval et al., 2006; El-Huneidi et al., 2006).

DNA Extraction

Bacterial DNA was extracted from overnight broth cultures of the various strains according to the method reported by Atashpaz et al., (2010) with some modification. The DNA extraction process was carried out as follows:

1) One milliliter overnight culture at 37°C in 5ml brain heart infusion (BHI) broth of each strain was transferred into a centrifuge tube and centrifuged at 3500 rpm for 10 min.

2) The supernatant was discarded and then 800 μl lysing buffer was added to the pellet, mixed thoroughly, the lysing buffer (2% CTAB (Merck, Hohenbrunn, Germany), 100 mM Tris-HCl (Merck, Darmstadt, Germany), 1.4 M NaCl (Merck, Darmstadt, Germany), 1% PVP (AppliChem, Darmstadt, Germany), 20 mM disodium salt of ethylenediaminetetraacetic acid (Na2EDTA; Merck, Darmstadt, Germany), 0.2% LiCl (Merck, Darmstadt, Germany). The pH was adjusted at ~8 for the solution used before autoclaving). Afterwards, the prepared sample was transferred to a 1.5 ml centrifuge tube.

3) The sample was incubated at 65°C for 30 min and gently shacked every 10min.

4) The sample was centrifuged at 10000 rpm for 5 min at 4°C.

5) The supernatant was transferred into a new tube and an equal amount of chloroform– isoamylalcohol (Merck, Darmstadt, Germany) (24: 1 vol/vol) was added. Then the tube was gently flipped several times.

6) The sample was centrifuged at 12000 rpm for 8 min at 4°C. The upper phase was then transferred into a new tube.

7) An equal volume of cold (~20°C) isopropanol (Merck, Darmstadt, Germany) was added to precipitate the DNA. Then the sample was stored at ~20°C for 30 min.

8) The sample was precipitated at 14000 rpm for 10 min at 4°C.

9) For the first washing step, after the removal of the supernatant, 500 μl of 96% ethanol (Merck, Darmstadt, Germany) (4°C) was added to the sample which was then centrifuged at 12000 rpm for 5 min.

10) For the second washing step, the supernatant was removed and 500 μl of 70% ethanol (4°C) was added to the sample which was then centrifuged at 12000 rpm for 5 min.

11) The supernatant was removed and the pellet was dried at room temperature.

12) The genomic DNA pellet was dissolved in 50 μl TE buffer [10 mM Tris-HCI (pH 8.0), 1 mM EDTA (pH 8.0), (Merck, Darmstadt, Germany)] and DNA solution was stored at ~20°C.
Assessing the Quantity and Quality of Extracted DNA

The quantity of the extracted DNA was checked by NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The A260/A280 absorbance ratio was used to determine undesired contaminations.

To evaluate the quality and intactness of the extracted DNA, gel electrophoresis was used. The extracted DNA (5 µl) was loaded on 1% agarose gel (Invitrogen, California, USA), which contained ethidium bromide (1 µg/ml) for DNA staining. And finally, a G:Box™ gel documentation system (Syngene, Cambridge, United Kingdom) was used for image acquisitions.

PCR Primer Design

Six PCR primer sets were used to detect the staphylococcal enterotoxin A gene (sea), staphylococcal enterotoxin B gene (seb), staphylococcal enterotoxin G gene (seg), staphylococcal enterotoxin H gene (seh), staphylococcal enterotoxin I gene (sei) and staphylococcal enterotoxin J gene (sej) as primers reported previously in the literature. The primers used in this study and their respective amplified products are listed in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Primer sequences</th>
<th>Size of amplified product</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>sea</td>
<td>SEA-f</td>
<td>GCA GGG AAC AGC TTT AGG C</td>
<td>521 bp</td>
<td>(Monday et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>SEA-r</td>
<td>GTT CTG TAG AAG TAT GAA ACA CG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seb</td>
<td>SEB-f</td>
<td>ACA TGT AAT TTT GAT ATT CGC ACT G</td>
<td>667 bp</td>
<td>(Løvseth et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>SEB-r</td>
<td>TGC AGG CAT CAT GTC ATA CCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seg</td>
<td>SEG-1</td>
<td>AAG TAG ACA TTT TTG GCG TTC C</td>
<td>287 bp</td>
<td>(Omoe et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>SEG-2</td>
<td>AGA ACC ATC AAA CTC GTA TAG C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seh</td>
<td>SEH-f</td>
<td>CAA CTG CTG ATT TAG CTC AG</td>
<td>360 bp</td>
<td>(Monday et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>SEH-r</td>
<td>GTC GAA TGA GTA ATC TCT AGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sei</td>
<td>SEI-1</td>
<td>GGT GAT ATT GGT GTA GGT AAC</td>
<td>454 bp</td>
<td>(Omoe et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>SEI-2</td>
<td>ATC CAT ATT CTT TGC CTT TAC CAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sej</td>
<td>SEJ-1</td>
<td>CAT CAG AAC TGT TGT TCC GCT AG</td>
<td>142 bp</td>
<td>(Monday et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>SEJ-2</td>
<td>CTG AAT TTT ACC ATC AAA GGT AC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Multiplex PCR Amplification

In the recent study, genomic DNA of S. aureus strains was amplified in one set of Multiplex PCR. Multiplex PCR reaction was performed in a final volume of 25µl, containing: 1µl of genomic DNA (50 ng/µl), 1 µl of Taq polymerase (5 unit/µl), 0.4 µM of each primers, 200 µM of each dNTP, 2.5 µl of 10X PCR buffer and 1 µl of MgCl₂ (1.5 mM).
The amplification program consisted of one initial denaturation at 94°C for 4 min followed by 32 cycles of 50 sec at 94°C for denaturation, 40 sec at 56°C for primer annealing, 180 sec at 72°C for extension and DNA synthesis and final extension at 72°C for 10 min. The products were separated on 1.5% agarose gel containing ethidium bromide (1 μg/ml), then images were taken using a G: Box™ gel documentation system (Syngene, Cambridge, United Kingdom).

RESULTS AND DISCUSSION

Results

In the present study, 40 *S. aureus* strains isolated from milk samples of cows with mastitis were tested for enterotoxin production by Multiplex PCR assay. Testing with specific primers for *sea*, *seb*, *seg*, *seh*, *sei* and *sej* genes were performed by which the existence of a 521bp segment was related to the amplification of a specific fragment of gene *sea* that is responsible for enterotoxin type A (Figure 1, lane 9). DNA amplification fragments of 667 bp for staphylococcal enterotoxin are related to B gene (*seb*) (Figure 1, lanes 1, 5 and 8), 287 bp for staphylococcal enterotoxin G gene (*seg*) (Figure 1, lanes 1, 5 and 8), 360bp for staphylococcal enterotoxin H gene (*seh*) (Figure 1, lanes 1, 4 and 5), 454 bp for staphylococcal enterotoxin I gene (*sei*) (Figure 1, lanes 2 and 8) and 142 bp to staphylococcal enterotoxin J gene (*sej*) (Figure 1, lanes 1, 5 and 8). Also, *S. epidermidis* was used as a negative control and did not yield a PCR product (Figure 1, lanes 3 and 7).

A total of 40% of the tested *S. aureus* isolates were positive for one or more toxin genes. 7.5 percent of total isolates were *sea* positive, 10% *seb* positive, 32.5% *seg* positive, 20% *seh* positive, 10% *sei* positive and finally, 22.5% *sej* positive.

![Figure 1: Multiplex PCR assays for the detection of enterotoxin genes in *S. aureus* strains](image)

Discussion

*S. aureus* is recognized worldwide as a major pathogen causing bovine mastitis, and milk from infected animals is considered to be the main source of enterotoxigenic *S. aureus* of animal origin (Zschock et al., 2005).

In the present study, we have described a Multiplex PCR-based diagnostic protocol to detect the genes for enterotoxins A, B, G, H, I and J in *S. aureus* isolated from milk samples of cows with mastitis. This technique can identify strains harboring the toxin genes and is independent of the expression and secretion of the toxin (Mehrotra et al., 2000).
Multiplex PCR results obtained in this study showed that 40% of *S. aureus* isolates were positive for one or more enterotoxin genes. This prevalence rate was much lower than 80.2% reported by De Freitas *et al.*, (2008) in the isolates of *Staphylococcus* spp. obtained from cows diagnosed with subclinical mastitis in Brazil. High frequencies of enterotoxin-encoding genes have also been reported in *S. aureus* isolates of bovine mastitis origin in studies carried out in Japan (71.4%) (Omoe *et al.*, 2002) and Italy (70%) (Vimercati *et al.*, 2006).

But, the percentage obtained in this study was more than what was reported in isolates from bovine mastitis in Turkey (29.3%) (Karahan *et al.*, 2009) and in Brazil (9.4%) (Da Silva *et al.*, 2005). The predominant enterotoxin type in this study was seg (32.5%). This has also been reported as the most frequent gene in *staphylococcus* from cows with subclinical mastitis by De Freitas *et al.*, (2008), who observed seg in 35% of strains. The seg gene was also reported in 31.4 % of strains by Wang *et al.*, (2009), amongst *Staphylococcus aureus* isolates from milk samples of bovine subclinical mastitis cases. The seg gene was detected in 22.5% of *S. aureus* isolates. It was reported in 24.4% of strains isolated from bovine mastitis by Wang *et al.*, (2009) and in 10% of strains by Unal (2013). The seh gene was detected in 20% of *S. aureus* isolates. It was reported in 32% of strains by De Freitas *et al.*, (2008) and in 5% of strains by Unal (2013).

The sei gene was detected in 10% of *S. aureus* isolates. This prevalence rate is lower than what was reported by Karahan *et al.*, (2009), who reported the detection of sei in 25% of strains, and Wang *et al.*, (2009), who found a prevalence of 31.8%. The sea and seb genes were detected in 7.5% isolates and 10% isolates, respectively. The sea gene, however, was not observed in the *S. aureus* investigated in some studies (Da Silva *et al.*, 2005; Karahan *et al.*, 2009; Omoe *et al.*, 2002). On the other hand, this gene is reported to be the predominant enterotoxin gene amongst *S. aureus* isolates of bovine mastitis origin in other studies (Wang *et al.*, 2009). The variation in reported rate results is probably due to geographical variations. Although the differences in study populations, sampling procedure, the number of samples studied, culture techniques might be effective (Becker *et al.*, 2003; Mehrotra *et al.*, 2000).

**Conclusion**

Multiplex PCR method was used to investigate the presence of sea, seb, seg, seh, sei and seg genes in *S. aureus* isolated from milk samples of cows with mastitis for the first time in Iran. Our results revealed that this method is simple, sensitive, low cost, relatively rapid and very specific and that it can identify several genes that encode toxin at the same time. Besides, on the basis of our examinations and available scientific literature, the conclusion reached was that *S. aureus* causing mastitis in cows can harbor enterotoxin-encoding genes with seg as the most frequent gene observed amongst the investigated isolates. These finding are important for surveillance purposes, since enterotoxin G should be investigated in human staphylococcal food poisoning outbreaks caused by the consumption of cow milk and dairy products.

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