MOLECULAR DETECTION OF VIRULENCE FACTORS TSST-1, ETA, ETB GENE IN STAPHYLOCOCCUS AUREUS ISOLATED FROM MILK AND MILK PRODUCTS

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ABSTRACT

*Staphylococcus aureus* causes a wide range of illnesses to humans and animals its consider common causes of food poisoning due to contaminated food product with this bacteria and its enterotoxin, so the current study was designed to identify *S. aureus* strains in random local samples of milk and cheese, about 120 (80 milk, 40 cheese) sample were subjected to microbiologically and molecular detection for the presence of Staphylococcus aureus, enterotoxigeny, DNA amplification showed that *S. aureus* isolates positively in 45 (37.5%) from total sample by PCR, 34 (42.5%) sheep milk, 11 (27.5%) sheep cheese.. By Multiplex PCR results showed the presence of virulence genes encoding toxic shock syndrome toxin (tsst-1) in 20 of sample and exfoliative toxins ETA, ETB gene 8,11 were detected the isolates respectively. Amplification of 16S rRNA gene using uniplex PCR with product size 228 bp while *Tsst-1* ETA ETB gene amplify 326 bp, 464 bp, 226 bp respectively. The results also showed a significant contamination with of *S. aureus*. It is concluded that must to minimize food poisoning, it is important for setting and maintain safety hygiene protocols.

**Key words:** *S. aureus*, milk, cheese, enterotoxigencity, Multiplex PCR.

I. INTRODUCTION

Food poisoning caused by *Staphylococcus aureus spp.* (SFP) is common an intoxication resulting from consumption of contaminated food with adequate levels of pre-formed enterotoxins. Ingesting 100-200 ng of SE in susceptible patients could cause staphylococcal Food Poisoning (SFP) (FDA, 2012) Symptoms of SFP poisoning could manifested within 2-8 hours of ingestion and include vomiting, nausea, abdominal cramping with or without diarrhea which typically resolve during 24-48 hr. (Argudin et al., 2010). Due to misdiagnosis and comparatively tiny outbreaks that are not reported, the percentage of people affected by SFP can only be estimated Hospitalization is rare, however it has been observed in immunocompromised patients, mainly the elderly and the very young. (Scallan et al., 2011), *Staphylococcus aureus spp.* considered as the third most important cause of food-borne illnesses in worldwide that affect 6 to 80 million people reach to 9,000 deaths each year (Tirado & Schmidt, 2001) its has vast diversity of virulence mechanisms that include pathogenicity, toxin production, and antimicrobial resistance (Argudin et al., 2010). Staphylococcal food poisoning was commonly transmitted via milk and milk products (Gensen et al., 2005). These products were among the foods most commonly linked to food-borne outbreaks (ANVISA, 2015). Because of its high moisture content and handling, raw milk and cheese are very susceptible to microbial and proteolytic change (Chalita et al., 2009). Several researchers have reported the significance of *S. aureus* in cheese made from buffalo and sheep milk and the potential for staphylococcal enterotoxins to cause food poisoning in humans (Johler et al., 2015). The supplierized These food to the underground market, in absent supervision or hygienic control. As a result of these factors, it was frequently implicated in the spread of pathogenic organisms and food poisoning. (Dorigon, 2010). Additionally, *S. aureus* isolated from foods of animal origin was showed resistance to several antimicrobial agents used to treat diseases, also its ability to produces a variety of protein toxins, including enterotoxins (SEs) toxic shock syndrome toxin1 (TSST-1), exfoliative These virulence factors, including super antigenic toxins, are may contribute to its pathogenicity (Boha et al, 2007). These toxins played an important role in staphylococcal disease and cause food poisoning. Because they are responsible for secreted certain proteins which can interact with antigen-presenting cells to induce cellular proliferation with high-level cytokine expression. However, some certain strains of *S. aureus* were produce one or
both of two immunologically unique exfoliative toxins (ETs), include ETA and ETB. also have been associated with a series of impetiginous staphylococcal diseases referred to as staphylococcal scalded skin syndrome. Although these two genes have identical bio-logical activity and a degree of genetic resemblance, the gene coding ETA is chromosomal, while ETB gene is plasmid linked. Many molecular epidemiological studies have already been con-ducted on enterotoxigenic S. aureus isolated from sheep milk, food (Scherrer et al., 2004).this experiment designed to isolate and identify S. aureus in milk and cheese in local market in Iraq and molecularly characterize the isolates for the presence of virulence genes enterotoxins (TSST-1, ETA, ETB) in the studied dairy samples.

II. MATERIAL AND METHODS

2.1 Sample collection
From May to August 2019, about 120 raw samples (cheese, milk) were collected from retail stores in Diwanhyia city, all samples transmitted by ice box (4 to 8°C) to the laboratory of microbiological and molecular analysis in College of in Veterinary Medicine, University of Al-Qadisiyah within 24 h. and kept at 4°C until analysis.

2.2 Isolation and identification of S. aureus:
Characterization of S. aureus was carried out as reported in (Normanno et al. 2007). for S. aureus enumeration (Downes et al. 2009) serial dilutions sample were homogenate and cultured on Baird Parker agar (Oxoid) supplemented with 5% egg yolk tellurite emulsion (Oxoid) then incubated at 35°C for 48 h the staphylococcal isolates. After this period, typical colonies appear circular, smooth - black, with off-white margin, surrounded by opaque zone, also subjected to phenotype after 24 or 48 h by using a VITEK system GP card (bioMerieux).

2.3. Molecular biology technique (PCR):
Table (1) Oligonucleotide Primers that have been used for the Amplification of the Genes Encoding Staphylococcal 16 rRNA and its Toxins

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide Primer Sequence</th>
<th>bp</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>16S rRNA</strong></td>
<td>GTAGGTGGCAAGCGTTATCC CGCACATCAGCGTCAG</td>
<td>228</td>
<td>(Puah et al., 2016)</td>
</tr>
<tr>
<td><strong>TSST-1</strong></td>
<td>F ACCCCTGTCCCTTATCATC</td>
<td>326 bp</td>
<td>(Rossato et al., 2018)</td>
</tr>
<tr>
<td><strong>ETA</strong></td>
<td>F TTTGCTTCTTGGATTGATTC R GATGTGTTCCGTTTGATTGAC</td>
<td>464 bp</td>
<td>(Kumar et al 2015)</td>
</tr>
<tr>
<td><strong>ETB</strong></td>
<td>F ACAAGCAAAAGAATACAGCG R GTTTTGGCTGCTTCTCTTG</td>
<td>226 bp</td>
<td>(Wang et al 2017)</td>
</tr>
</tbody>
</table>

2.3.1. PCR: detection of toxin genes

**Genomic DNA extraction:** DNA was extracted from overnight brain heart infusion broth. The DNA extraction procedure was done as described previously (Brakstad, Mæland & Tveten 2009)). DNA Template were dissolved in Tris-EDTA buffer (10 mM Tris chloride, 1 mM EDTA (pH 8) the concentration was determined as μg/mL. according to A260 values. The amount of Template DNA ranging from 10 to 1,000 ng. wo PCR reactions First one for amplify 16 rRNA gene, second reactions multiplex PCR for amplify ETA, ETB, and TSST-1, according to (Holtfreter et al.2007) and (Lovseth et al.2004) with modifications. ETA, ETB, AND TSST-1 gene The primer sequences used in this study described in ( Table 1) both reactions were performed in a total volume of 25 μL containing reaction buffer (5×; 10 mM Tris- HCl, pH 7.5, 1 mM EDTA, pH 8) 100 mM of dNTP, 5.0 mM MgCl2, 150 to 400 nM of each primer, 0.1 U from Taq DNA polymerase, and 10 to 20 ng of DNA template. The amplification PCR samples done by using a thermocycler (Axygen, Tewksbury, MA) program as following: 1-PCR mixture for all genes amplification conditions were prepared and applied as a total of 35 cycles were performed as following initial denaturing step at 92°C for 2 min annealing at 54°C for 1 min, and A last step of extension at 72°C for 3 min. After the initial denaturing step at 94°C for 5 min, annealing at 56°C for 1 min, in end extension step at 72°C for 5 min was applied PCR amplicons were detected by electrophoresis in 1.5 % (w=v) agarose gel and stained with ethidium bromide and then viewed under an ultraviolet transilluminator for 90 min.
III. RESULTS

Table 2. Prevalence of S. aureus and ETB, ETA, TSST-1 genes in positive sample isolated from milk and cheese (n=120)

<table>
<thead>
<tr>
<th>Type samples</th>
<th>isolates (%)</th>
<th>ETB %</th>
<th>ETA %</th>
<th>TSST-1 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw sheep milk 80</td>
<td>34 (42.5 %)</td>
<td>6 13.3%</td>
<td>8 17.7%</td>
<td>13 28.8%</td>
</tr>
<tr>
<td>Sheep cheese 40</td>
<td>11 (27.5 %)</td>
<td>2 4.4%</td>
<td>3 6.6%</td>
<td>7 15.5%</td>
</tr>
<tr>
<td>Total dairy products 120</td>
<td>45 (37.5%)</td>
<td>8 17.7%</td>
<td>11 24.4%</td>
<td>20 44.4%</td>
</tr>
</tbody>
</table>

Figure 1: PCR amplification for the S. aureus 16 r RNA gene at 228 bp the Lane 1: DNA molecular marker (100-bp ladder). Lanes 2, 3, 5, 7, 8, 9, 10: positive samples.

Figure 2: Results Multiplex PCR amplification for the S. aureus enterotoxin genes. Lane 1: DNA molecular marker (100-bp ladder). Lanes 3, 5, 7, 8, 9 and 12: positive samples for ETA gene at 464 bp. Lanes 4, 6, 7, 10 and 11: positive samples for TSST-1 gene at 326 bp. Lanes 3, 6, 7 and 9: positive samples ETB gene at 226 bp.
IV. DISCUSSION

From 120 milk and cheese samples were analyzed, prevalence of *S. aureus* was identified in 45 (37.5%) All strains were identified by phenotypic characterization, and confirmed by PCR amplification of the 16S rRNA gene with product size 228 bp for intergenic spacer region. all samples collected over the period of the experiment basis of the results obtained regarding the culinary and biochemical properties *S. aureus* presents in raw milk and cheeses were frequently finding around the world. Several authors have also reported that most common prevalence of *S. aureus* isolated from (Dakic et al; 2005) in dairy product for example, 35.0% in Italy (Carfora et al., 2015), 45.0% in Brazil (Ferreira et al., 2016), while in Sweden 69.0% (Rosengren et al., 2010). The locally handmade process of milk and cheeses in iraq does not involve milk pasteurization, which is essential role for the quality control and for prolong the shelf life of this product, also staphylococcal food poisoning outbreaks reported by (Baumgartner, 2008) were associated with cheese consumption, multiplex PCR technique was performed to detect virulence genes that coding production of toxic shock syndrome toxin (TSST-1), and exfoliative toxins (*ETA* & *ETB*), with amplification of (326 bp, 464 bp, 226 bp bp) respectively the result showed overall, 20 isolation found positive as coding genes (*TSST-1* gene) and 8, 11 for *ETA*, *ETB* respectively its is came similar to the results obtained by (Lue et al., 2014), and (Bianchi et al. 2014) when evaluating milk products found genes *TSST-1*, in 23 sample 12.9% of the isolates, as well as the genes *ETA*, *ETB* in 8.11, respectively enterotoxin genes *ETA*,*ETB* and *TSST-1* The prevalence of enterotoxigenic *S. aureus* isolates had increased, demonstrating that the bacteria’ hazardous ability had increased (Bianchi et al., 2014). Most concern toxins of *S. aureus*, enterotoxins because when it produced can cause food poisoning, which is one of the most prevalent foodborne illnesses in worldwide (ECDC, 2015). The responsibility of staphylococcal about food poisoning involving dairy products described as an outbreak due to consumption of milk contaminated with enterotoxigenic *S. aureus* producing(Ostyn et al., 2010), while (Jørgensen et al., 2005) reported Another outbreak was attributed to produce by *S. aureus* toxins in mashed potatoes made with raw milk. In collocation the presence of enterotoxins in dairy products was considered a potential risk to public health, a Variety of factors associated with *S. aureus* multiplication and enterotoxin production (Saadat et al., 2014), like processing failures, improper refrigeration, poor hygiene procedures, and contamination after processing Therefore, the establishment and implementation of safety regulations is necessary to prevent staphylococcal food poisoning.

V. CONCLUSION

The high incidence of *S. aureus* in raw milk and cheese in this study is significant because it indicates a potential risk to public health. Beside to the detection of toxin-encoding genes, which confirms the product's probable source of contamination and reinforces the need to limit contamination levels by strictly controlling and measuring the product's production and consumption. The virulence characteristic observed in the most of the isolates leads to conclude that, notwithstanding the handcrafted manufacture, *S. aureus* contamination of traditional and handmade raw milk and cheese is most likely of animal origin. As a conclusion, milk pasteurization, as advised which should be eliminate the majority of the problem.

REFERENCES


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