Prevalence of virulence genes, agr and antimicrobial resistance of
Staphylococcus aureus isolated from food and dairy products in
Hamadan, Iran

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ABSTRACT

Staphylococcus aureus is one of the most important foodborne pathogen in food products. Exfoliative toxins (ETs) and toxic shock syndrome toxin-1 (TSST-1) are important virulence factors that facilitate bacterial attack and reproduction in the body of host. Expression of exfoliative toxins controlled by accessory gene regulator (agr) locus. Therefore, the identification of these genes in Staphylococcus aureus in food samples is very necessary and important. The aim of this study was prevalence of virulence genes (tsst1 and ETs) and agr of Staphylococcus aureus isolated from food products and association of these genes with antibiotic resistance. Between 2013 and 2014, 1050 food samples of various origin purchased and collected in Hamadan province in Iran. The S. aureus strains were isolated from raw milk and dairy products (n= 671) and raw meats (n=379). Samples were examined for the presence of S. aureus. Strains were characterized using standard microbiological procedures. Molecular identification of S. aureus strains confirmed by PCR. All isolates were screened for tsst1, agr, etc genes by PCR amplification. Out of 1050 samples, 98 (9.33%) samples were positive for S. aureus, including 36 (9.49%) of 379 raw meats and 62 (7.2%) of 671 raw milk and dairy products. The most frequent resistance was observed to erythromycin (30.6%), followed by tetracycline (29.6%), Gentamicin (27.6%), Clindamycin (26.5%), Ciprofloxacin (24.5%), Rifampin (24.5%), Sulfamethoxazole/Trimethoprim (14.3%), and Cefoxitin (6.1%). The TSST-1 was identified in 30.61 percent of isolates, while the eta and etd were found in 63.26% and 75.51 percent of isolates, respectively. The distribution of agrA and agrC genes among the 98 food isolates were 63.26 and 14.28 percent, respectively. The detection of the high prevalence rate of virulence genes in this study indicates a potential risk for causing animal originated food poisoning that is a serious problem for public health. Infected animals and acquisition of infection during the processing stage are the main causes of contamination with S. aureus. Therefore, continuous surveillance is essential for monitoring of pathogens that are capable of causing food poisoning.

Keywords: Staphylococcus aureus, TSST-1, ETs, age, Antibiotic resistance

INTRODUCTION

The percent of the diseases due to foodborne pathogens remains largely unknown and approximately two thirds foodborne illness were caused by unknown agents[1, 2]. Staphylococcus aureus is one of the most important foodborne pathogen in food products[3]. This pathogen produce various infections from relatively mild to more severe diseases[4]. These infections very difficult to treat due to the development of drug resistance, especially methicillin-resistant S. aureus (MRSA)[5]. In addition, this microorganism has enough potential to contaminate animal products such as meats, milk and dairy products[6, 7].
S. aureus produces many toxins such as toxic shock syndrome toxin 1 (TSST-1) and exfoliative toxins (ETs) that facilitate bacterial attack and reproduction in the body of host[8]. TSST-1 is considered to be a superantigen that role in the Staphylococcal Toxic Shock Syndrome (TSS)[9]. TSS is characterized by high fever, headache, confusion, subcutaneous oedema, rash, desquamation, diarrhea, vomiting and hypotension, that resulting in multiple organ failure[10]. Secreted superantigens at mucosal sites such as vagina and nasopharynx lead to a massive release of cytokines including tumor necrosis factor (TNF), interleukin-2 (IL-2) and IFN-γ which are responsible for development of the typical clinical signs[11]. Exfoliative toxins(ETs), responsible for staphylococcal scaldedskin syndrome(SSSS)[12]. The genes encoding ETs (eta, etb, and etd) are located on mobile genetic elements (MGEs) such as prophages, plasmids or transposons[13, 14]. Transfer of these genes through MGEs involve in distribution of pathogenic strain of Staphylococcus aureus and emergence of super bugs[15]. ETs have serine protease activity and cleave a peptide bond in the extracellular region of desmoglein 1 (Dsg1) and facilitating bacterial skin invasion[16]. In S. aureus the expression of most virulence factors and surface proteins controlled by accessory gene regulator (agr) locus which encodes a two-component system that down-regulate surface proteins and up-regulate secreted proteins[17]. Based on study of Foster in 1987, expression of gene encoding ETA(eta) in S. aureus is dependent on agr locus[18]. It seems that agr locus have a major role in human infection. It has been suggested that changes in the secretion of virulence factor expression can lead to acquisition of antibiotic resistance in S. aureus[19-21].

The aim of this study was prevalence of virulence genes (tsst1, agr and eta) of Staphylococcus aureus isolated from food products and association of these genes with antibiotic resistance.

MATERIALS AND METHODS

2.1. Food samples and bacterial strains
Between 2013 and 2014, 1050 food samples of various origin purchased and collected in Hamadan province in Iran. The S. aureus strains were isolated from raw milk and dairy products (n= 671) and raw meats (n=379). Samples were examined for the presence of S. aureus. Strains were characterized using standard microbiological procedures (Gram-staining, hemolytic activity on sheep blood agar, catalase production, oxidase test, growth in Baird–Parker agar and coagulase tube test). In addition, molecular identification of S. aureus strains confirmed by PCR.

2.2. Antimicrobial susceptibility testing
The antibiotic susceptibility pattern of all isolates was determined by disk agar diffusion method with the zone diameters measured at 24 h according to the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2013). Mueller Hinton agar was used for antimicrobial susceptibility test. The 8 antibiotic discs (Mast, UK) were Cefoxitin (30µg), Ciprofloxacin (5µg), Clindamycin (2µg), Erythromycin(15µg), Gentamicin(30µg), Rifampicin(5µg), Trimethoprim/ Sulphamethoxazole(1.25/23.75µg) and Tetracycline (30µg). Strain ATCC 25423 was used as positive control.

Interpretation of results were applied according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

2.3. DNA extraction and PCR for tsst1, agr and eta genes
Total DNA was extracted from overnight-grown pure isolates using the DNA extraction Kit (BioFlux Co., Tokyo, Japan), according to the manufacturer’s instructions. Extracted DNA stored at 40°C for use in PCR amplification. All isolates were screened tsst1, age, eta genes by PCR amplification. All primer sequences have been listed in Table 1. The genes encoding agr (agrA, agrB, agrC and agrD), toxic-shock syndrome toxin-1 (tsst1) and exfoliative toxin genes (eta and etd) were detected by PCR. PCR products were visualized by UV transillumination after electrophoresis on 1.0% agarose gels.
Table 1: Oligonucleotide primers used in PCRs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
<th>Product (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuc</td>
<td>F: GCGATTGATGTATCGAGGTT&lt;br&gt;R: AGGCGGAATATGACCTTGAAAGAATGACGAGTCGAAGTTGCT</td>
<td>279</td>
<td>[22]</td>
</tr>
<tr>
<td>TSST-1</td>
<td>F: PTACTATTGTGAAAGTGTCACTATGTT&lt;br&gt;R: TTTGACATGATCTTCTTGTCACTCATACG</td>
<td>180</td>
<td>[23]</td>
</tr>
<tr>
<td>eta</td>
<td>F: ACTGTAGGAGCTAGTGCATTTGT&lt;br&gt;R: TGGATACTTTTCTATTTTCTATTTTCTATTTTCTATTTTCT</td>
<td>190</td>
<td>[23]</td>
</tr>
<tr>
<td>etd</td>
<td>F: GAAATAGTACCGCGCTAAATAATATG&lt;br&gt;R: CGGTATTTTCTTCTCGGAGAATG</td>
<td>492</td>
<td>[23]</td>
</tr>
<tr>
<td>agrA</td>
<td>F: GTGACAAAGTATATAAGGTTGCGAT&lt;br&gt;R: GTATTTCTAATCGAAAGTTCGCTATAGC</td>
<td>440</td>
<td>[24]</td>
</tr>
<tr>
<td>agrB</td>
<td>F: GTCACAAAGTATATAAGGTTGCGAT&lt;br&gt;R: GTATTTCTAATCGAAAGTTCGCTATAGC</td>
<td>572</td>
<td>[24]</td>
</tr>
<tr>
<td>agrC</td>
<td>F: GTGACAAAGTATATAAGGTTGCGAT&lt;br&gt;R: CTGGTGAAGGAAAGGTCACACTAAAAAGGTCCT</td>
<td>406</td>
<td>[24]</td>
</tr>
<tr>
<td>agrD</td>
<td>F: GTGACAAAGTATATAAGGTTGCGAT&lt;br&gt;R: GTGATCGGTAATACCGGCGCGAAGATGTTGTTGACT</td>
<td>588</td>
<td>[24]</td>
</tr>
</tbody>
</table>

2.4. Statistical analysis
All data analyses were performed using the statistical software SPSS16. Statistical analysis was performed using the Pearson Chi-squared test to calculate the strength of association between the virulence genes and origin of the isolates, as well as between virulence genes and antibiotic susceptibility patterns. A value of \( P < 0.05 \) was considered significant.

RESULTS

3.1. Isolation and identification of S. aureus
Out of 1050 samples, 98 (9.33%) samples were positive for S. aureus, including 36 (9.49%) of 379 raw meats and 62 (7.2%) of 671 raw milk and dairy products. Among food products, traditional cheese with 19 (11.17%) isolates from 170 samples and cream with 2 (4.25%) isolates from 47 samples had the highest (19) and the lowest S. aureus infection, respectively. For molecular confirmation of S. aureus strains, PCR amplification for nuc gene was performed. All 98 strains carried nuc gene.

3.2. Identification of TSST-1, eta, agr genes
DNA of isolates was examined for the presence of TSST-1, eta, agr genes. The TSST-1 was identified in 30.61 percent of isolates, while the eta and etd were found in 63.26 and 75.51 percent of isolates, respectively. The distribution of agrA and agrC genes among the 98 food isolates were 63.26 and 14.28 percent, respectively. Results had shown in Table 2.

Table 2: Prevalence of TSST-1, eta and agr genes in S. aureus isolated from food products

<table>
<thead>
<tr>
<th>Types of samples</th>
<th>Sample size</th>
<th>No of S. aureus isolates (%)</th>
<th>TSST-1 (%)</th>
<th>eta (%)</th>
<th>etd (%)</th>
<th>agrA (%)</th>
<th>agrC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>271(25.8)</td>
<td>29(10.7)</td>
<td>10(10.2)</td>
<td>16(16.32)</td>
<td>24(24.86)</td>
<td>16(16.32)</td>
<td>6(6.12)</td>
</tr>
<tr>
<td>Cheese</td>
<td>170(16.19)</td>
<td>19(11.17)</td>
<td>6(6.12)</td>
<td>14(14.28)</td>
<td>14(14.28)</td>
<td>14(14.28)</td>
<td>4(4.08)</td>
</tr>
<tr>
<td>Yogurt</td>
<td>45(4.28)</td>
<td>2(4.44)</td>
<td>0(0)</td>
<td>2(2.04)</td>
<td>0(0)</td>
<td>2(2.04)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Cream</td>
<td>66(6.28)</td>
<td>4(6.06)</td>
<td>2(2.04)</td>
<td>2(2.04)</td>
<td>2(2.04)</td>
<td>0(0)</td>
<td>2(2.04)</td>
</tr>
<tr>
<td>Skim</td>
<td>47(4.47)</td>
<td>6(12.76)</td>
<td>0(0)</td>
<td>2(2.04)</td>
<td>2(2.04)</td>
<td>2(2.04)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Butter</td>
<td>72(6.85)</td>
<td>2(2.77)</td>
<td>2(2.04)</td>
<td>4(4.08)</td>
<td>4(4.08)</td>
<td>2(2.04)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Meat</td>
<td>243(22.94)</td>
<td>25(10.28)</td>
<td>8(3.33)</td>
<td>14(14.28)</td>
<td>20(20.4)</td>
<td>16(6.32)</td>
<td>2(2.04)</td>
</tr>
<tr>
<td>Chicken</td>
<td>136(12.95)</td>
<td>11(8.08)</td>
<td>2(2.04)</td>
<td>8(6.15)</td>
<td>8(6.15)</td>
<td>10(10.2)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Total</td>
<td>1050(100)</td>
<td>98(9.33)</td>
<td>30(30.61)</td>
<td>62(63.26)</td>
<td>74(75.51)</td>
<td>62(63.26)</td>
<td>14(14.28)</td>
</tr>
</tbody>
</table>

3.3. Antimicrobial susceptibility testing
A total 98 (9.33%) S. aureus strains from 1050 food samples were isolated. Among the 98 isolates, resistance to Erythromycin (30.6%) was the most frequently observed, Tetracycline (29.6%), Gentamicin (27.6%), Clindamycin (26.5%), Ciprofloxacin and Rifampin (24.5%), Trimethoprim-Sulfamethoxazole (14.3%), and Cefoxitin (5.1%). Results had shown in Table 3.
Table 3: Results of Antimicrobial susceptibility testing

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Sensitive No.(%)</th>
<th>Intermediate No.(%)</th>
<th>Resistant No.(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefoxitin</td>
<td>93 (94.9)</td>
<td>nd</td>
<td>5 (5.1)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>65 (66.5)</td>
<td>3 (3.1)</td>
<td>30 (30.6)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>68 (69.4)</td>
<td>1 (1.0)</td>
<td>29 (29.6)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>71 (72.4)</td>
<td>nd</td>
<td>27 (27.6)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>71 (72.4)</td>
<td>1 (1.0)</td>
<td>26 (26.5)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>74 (75.5)</td>
<td>nd</td>
<td>24 (24.5)</td>
</tr>
<tr>
<td>Rifampin</td>
<td>70 (71.4)</td>
<td>4 (4.1)</td>
<td>26 (26.5)</td>
</tr>
<tr>
<td>SXT</td>
<td>82 (83.7)</td>
<td>2 (2.1)</td>
<td>14 (14.3)</td>
</tr>
</tbody>
</table>

nd: not detected

3.4. Statistical analysis
The statistical analysis showed that there was a significant relationship between origin of the isolates and agrA gene as well as between origin of the isolates and resistance to Erythromycin, Tetracycline, Clindamycin, Gentamicin, Ciprofloxacin, Rifampin and SXT (p<0.05). A statistically significant correlation was found between some antibiotic susceptibility patterns and carrying virulence genes in examined isolates. This included between Erythromycin and carrying eta genes, between Clindamycin and carrying agrA genes, and between SXT and carrying eta genes (p<0.05).

DISCUSSION
Exfoliative toxins(ETs), toxic shock syndrome toxin-1(TSST-1) can horizontally transfer between the strains and accessory gene regulator (agr) have a significant role in expression of gene encoding ETA(eta) in S. aureus[25]. Food products have an important role in transfer and dissemination of these genes. The aim of this study was to determine the prevalence of TSST-1, eta and agr genes and antibacterial susceptibility pattern of S. aureus isolated from raw meats, raw milk and dairy products in Hamadan, Iran.

In our study, 9.33% of samples were contaminated by S. aureus; similar to a study performed in Tehran and Turkey, which reported contamination rates of 9.5% and 13.8%, respectively[26, 27]. In the other study, higher contamination rates of S. aureus have reported[28]. These discrepancies may be due to differences in ecological origin of the strains, cheese production process, number of colonized food handlers, level of hygiene and transport systems.

In this study the TSST1 was found in 30.61% of the S. aureus isolates. However, there are some studies with lower frequency (11%)[29]. In another study, the tsst1 gene not found in any of the strains of the isolates[12].

ETA, ETB, and ETD are three main human active exfoliative toxins that responsible for staphylococcal scalded skin syndrome. In our study, prevalence of eta and etd genes in the isolates were 63.26 and 75.51, respectively. None of the S. aureus isolates contained etb genes. These results were inconsistent with previous studies. In study of Minghui et al. etb and etd were not found in any isolate and eta was only detected in one isolate of 117 S. aureus food isolates[12]. In study of Aydin et al. also none of the S. aureus isolates contained eta and etb genes[27].

Karsten et al investigated 429 isolates of staphylococcus aureus and eta and etb gene were identified in 1.2 and 0.5 percent of them, respectively[30].

In the study carried out in Japan, it was demonstrated that ETA was present in 40 percent of strains, while 25 percent of cases contained ETB, and in five percent of them both genes were observed[31].

Moreover, in the study carried out by Osamu et al eighty-eight isolates were positive for eta alone, 25 were positive for etb alone, and 85 were positive for both genes[32].

These finding obtained by various studies conducted in this area stresses that the prevalence of these genes are different in different geographical regions.
For detection of agr types, we used four pair’s oligonucleotide primers. Among the agr genes, the agrA (75.51%) was the most prevalent followed by agrC. All isolates were negative for agrB and agrD genes. These results were similar to previous studies that agrA was prevalent in the meat and milk samples [33, 34]. In another study, all strains were non-typeable for agr locus [35]. This discrepancy may be due to geographic distribution and origin of the samples.

CONCLUSION

*S. aureus* is the most frequently isolated bacterium that causes many cases of food poisoning in the world. The detection of the high prevalence rate of virulence genes in this study indicates a potential risk for causing animal originated food poisoning. Increase of these genes in food products is a serious problem for public health. Infected animals and acquisition of infection during the processing stage are the main causes of contamination with *S. aureus*. Therefore, continuous surveillance is essential for monitoring of pathogens that are capable of causing food poisoning.

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REFERENCES