High rate of contamination with *Staphylococcus aureus* in traditional Koozeh cheeses. A molecular typing approach

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Parole chiave: *Staphylococcus aureus*, formaggi Koozeh, Enterotossine, Intossicazione alimentare, latticini

Abstract

Background. Koozeh cheese is an Iranian dairy product in rural areas, it is necessary to consider the microbial contamination in this supply.

Objective. This study evaluates microbial contamination in Koozeh cheese by molecular tools.

Material and Methods. *S. aureus* and its enterotoxins including type A and type B were identified by biochemical and polymerase chain reaction (PCR) and molecular typing was done by RAPD (Random Amplification of Polymorphic DNA) method. A total of 42 sheeps and cows Koozeh cheese samples were collected from random market in the cities and the surrounding villages.

Results. 71.42% of samples were contaminated with *Staphylococcus* spp. and in 50% of isolates, *S. aureus* specific coagulase gene “coa” was detected. High-level contamination was observed in 7.14% of samples. The SEA or SEB enterotoxins were produced in 42.84% of isolates. No clonal relationship was observed by molecular approach.

Conclusion. The obtained results indicate a high level of microbial contamination in Koozeh cheese. Half of isolates were enterotoxin producer and had high diversity and no clonal relationship. Long processing and manipulation are involved in contamination. Improvement in hygiene, training local manufactures of Koozeh cheese, control of products for possible contamination and developing new protocols is needed to decrement of *S. aureus* contamination in Koozeh Cheese products.

Introduction

*S. aureus* is a most common opportunistic bacterium in human pathogenicity (1), and the third most common pathogen of food poisoning throughout the world as well. It is coagulase-positive and this feature distinguishes it from the other *Staphylococcus* spp. *S. aureus* is able to produce a wide range of extracellular toxins and virulence factors, which contribute to disease procedure (2). Resistance to antibiotics is considered as a virulence factor in *S. aureus*. The strains of this bacterium can be resistant to one or more...
types of antibiotics and represent a major threat to public health (3). Staphylococcal food poisoning is related to consumption of enterotoxin-containing foods (4).

Some strains of *S. aureus* may produce up to 20 different serological staphylococcal enterotoxins (SEs), which induce the food poisoning. Among them, 11 principal antigenic types of SEs have been known (5). Symptoms are included vomiting, nausea, abdominal cramps and diarrhea, the appearance of disease in 2-4 hours after ingestion of food. The severity of disease depends on how much poison entered to body and body resistance. To incidence of food-borne poisoning, *Staphylococcus* spp. should be able to grow and produce enterotoxin in food supplies. Sometimes the amount of produced SE may be scrumpy for food intoxication. Moreover, many factors including temperature, salt concentration, pH, climatic conditions and the antagonistic activity competitive flora are involved in production of enterotoxin in foods (6).

One of the Virulence factors of *S. aureus* is resistance to antibiotics. Numerous bacteria are resistant to antibiotics because of the widespread prescription and misuse of antibiotics in human treating and animal diseases. In recent years, antibiotic resistance in bacteria causing concern about public health.

Increasing bacterial resistance and most infections caused by them, have attracted attention of researchers (7). Infections caused by methicillin-resistant *S. aureus* (MRSA), has become one of the great problems of antibiotic treatment (8). The first strains of MRSA were detected in humans and livestock in 1961 and 1972 respectively. This strain is resistant to many antibiotics, including beta-lactams, semi-synthetic penicillin, cephalosporins, Carbapenems and penems (9).

The important sources of contaminating foods to *S. aureus* are hands and nose of people who are working in the food production. Moreover, *S. aureus* is one of the major sources of pollution in dairy products, which cause breast inflammation in animals. It is often found in raw milk and was isolated from the skin of the breast, wounds and mucous membranes, milking equipment and cabinets, floor, door handles, etc. (10, 11). So its presence in raw milk and dairy products is an important concern for the health and the quality of traditionally produced cheeses (12).

White brine cheese, which is traditionally produced across Iran. This brine cheese is made by natural micro flora of milk from unpasteurized cow’s milk, sheep’s milk or mixture of both milks (13). The aim of this study was to determine the prevalence of coagulase positive *S. aureus* in traditional cheese in region of East-Azarbajian–Iran, Their Enterotoxin production, Molecular relation and antibiotic resistance. RAPD (Random Amplification of Polymorphic DNA) method was used to study clonality of the isolates from cheese samples.

**Materials and methods**

1. **Sample collection**

   In this study, 42 samples of cheese were collected randomly from the traditional market in the cities and villages of Azarbaijan-Iran. Selected samples with disposable glove were placed in sterile container, then samples were immediately transferred to laboratory and preserved in the refrigerator.

2. **Isolation of S. aureus**

   About 25 g of each cheese was added to 225 mL of sterile sodium citrate solution in a sterile stomacher bag separately. Following this, 0.1 ml of decimal dilutions of sample suspensions were streaked (seeded) aseptically onto sterile Mannitol Salt Agar (Merck, Germany), blood agar (Merck) and incubated at 37°C for 24-48 h under aerobic culture conditions. Then the colony description characteristics were investigated
based on Gram staining, catalase, DNase Test. DNase Test and all suspected colonies were evaluated by coagulase test using Bactident rabbit plasma (Merck).

3. Antimicrobial susceptibility testing

Antibiotic resistance of *S. aureus* isolates was performed by standard disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI 2016) guidelines using Mueller-Hinton agar (14). After inoculation of Mueller–Hinton agar plates with the *S. aureus* isolates, the antimicrobial discs were applied on the bacterial culture plates and incubated at 30 °C for 24 h (15). The used antimicrobial disks were as follow: gentamicin (10 μg), cefoxitin (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), novobiocin (30 μg), vancomycin (30 μg), oxacillin (1 μg), rifampicin (5 μg) and erythromycin (5 μg) (HiMedia Laboratories).

4. DNA extraction

In order to preparation of tissue buffer, 0.4 g of sodium hydroxide along with 0.5 g of SDS was added in 200 ml of deionized water. Then 20 micro-liters of tissue buffer added to reaction cup (Eppendorf) and one of bacterial colonies (as a colony) were dissolved into it. Eppendorf was placed in a hot plate for 10 minutes at 95 °C and after this step the mixture was centrifuged for 1 min at 13,000 × g (16).

5. PCR

To perform the PCR, 18 microliters of prepared mastermix with 2 micro-liters of extracted DNA were placed in a thermocycler device (Biorad, Singapore). To detect of coa genes in *S. aureus* isolates, PCR was performed according to previously described method (17). The sequence of primers used for amplification of coa gene was forward: 5′-ATA GAG ATG CTG GTA CAG G-3′ and Reverse: 5′-GCT TCC GAT TGT TCG ATG C-3′ (17). Primers for *S. aureus* Enterotoxins (SEA and SEB) were general Forward primer of: SE-U 5-TGT ATG TAT GGA GGT GTA AC-3 and reverse for SEA-R: 5-ATT AAC CGA AGG TTC TGT-3 and reverse of SEB-R: 5-ATA GTG ACG AGT TAG GTA-3. Product size for SEA was 270bp and for SEB was 165bp. *S. aureus* ATCC® 25923™ was used as standard control for coa gene.

Primers were prepared according to manufacturer’s instructions (Cinagen co, Iran, Tehran) so that initial powder primer was added to the required amount of sterile distilled water to reach primer concentration to 100 mM, thus prepared original primer Stoke was kept frozen at -20. used Mastermix includes PCR Buffer (2 μl), MgCl₂ (0/6 μl), dNTP (0.2 μl), primer Reverse (0.4 μl), primer Forward (0.4 μl), deionized water (13.9 μl), taq DNA polymerase (0.9 μl).

The PCR cycling protocol was applied as follow: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 second, annealing at 55°C for 45 second and extension at 72°C for 2 min, followed by a final extension at 72°C for 7 min (18).

6. Agarose Gel Electrophoresis and Visualization of PCR Products

The PCR products were evaluated by electrophoresis on a 1.5% standard agarose gel (Cinaclone, Tehran, Iran) in Tris-borate-EDTA buffer, and 100-bp DNA ladder was used as molecular marker. The samples were mixed in ratio of 3:1 with the loading buffer and was loaded into the wells. Electrophoresis was performed at a voltage of 100 V for 90 minutes (19)_ENREF_19.

After completion of electrophoresis for preliminary results and staining with Safestain (Yektatajhiz azma, Tehran, Iran) the gels were placed in a UV Transilluminator, in case visible band, the gels were placed inside Gel document and were photographed by a digital camera.
7. ETR molecular typing

The genetic similarity of isolates was examined by RAPD molecular typing method. PCR reaction was done in 25 ml mixtures consisting of 1× PCR buffer, 2.5 mM dNTP (Cinaclone, Iran), 5.0 μm of each RAPD primers (as per described previously (20)), 50 ng of template DNA and 3U Taq DNA polymerase (Cinaclone, Iran). PCR reaction was carried out by using a thermal cycler (Biorad, Australia) with cycling profile: initial denaturation at 95°C for 2 min, 45 cycles of amplification (denaturation at 94°C for 1 min, annealing at 32°C or 34°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 5 min. After electrophoresis, Data were compared by Gel compare software (Applied Maths, Belgium). Company for 30-day trial provided software. S. aureus ATCC® 25922™ was used as standard strain for comparisons.

Statistical analyses were performed via SPSS software version 21.0 (SPSS, Chicago, IL, USA). The variables were analyzed by univariate analysis using Chi square test. Statistical significance was set at 0.05.

Results

All cheese samples were cultured for S. aureus isolation in blood agar and MSA media. Most of the samples (71.42%) were contaminated with Staphylococcus spp. and S. aureus specific coA gene was detected in 50% of isolates (Table 1). Among the 42 cases, 3 samples (7.14 %) were found to be contaminated with S. aureus with higher than 100000 cfu (colony forming unite)/gram. Cow cheeses were more contaminated including 60% positive culture and 61.9% of positive cultures had specific gene (Table 1). About 9 (42.84%) of isolates produced SEA or SEB or both of them (Table 1). Comparing the results about presence of SEA, SEB and SEA+SEB producers in S. aureus isolates between cow and sheep cheese show any significant relationship (p= 0.548, 0.421, 0.585, respectively).

The results of antibiogram test for S. aureus isolates which were positive in the PCR showed no significant resistance to antibiotics. The S. aureus isolates were not resistant to any of antibiotics and only one of the isolates showed resistance to ciprofloxacin. There was no MRSA isolate among our studied samples.

Molecular genotyping showed no similarity or clonal diversity between isolates (Figure 2). All isolates were clustered separately and we did not identify any cluster with more than one member. Isolates similarity were less than 60% and only two isolates were similar with more than 70% genetic homogeneity.

Table 1 - Rate of contamination of Koozeh Cheese samples after culture in Mannitol Salt Agar (MSA) medium

<table>
<thead>
<tr>
<th>Cheese samples</th>
<th>No. of samples (%)</th>
<th>Growth on MSA⁴ (%)</th>
<th>coA gene positive (%)</th>
<th>SEA¹ (%)</th>
<th>SEB¹ (%)</th>
<th>SEA+SEB¹ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep cheese</td>
<td>17(40.47)</td>
<td>12(40)</td>
<td>8(38.09)</td>
<td>1(12.5)</td>
<td>0</td>
<td>2(25)</td>
</tr>
<tr>
<td>Cow cheese</td>
<td>25(59.52)</td>
<td>18(60)</td>
<td>13(61.9)</td>
<td>3(23.07)</td>
<td>1(7.69)</td>
<td>2(15.38)</td>
</tr>
<tr>
<td>Total‡</td>
<td>42(100)</td>
<td>30(71.42)</td>
<td>21(50)</td>
<td>4(19.04)</td>
<td>1(4.76)</td>
<td>4(19.04)</td>
</tr>
</tbody>
</table>

* MSA: Mannitol salt Agar Medium.
* In this row, percentages are calculated in compared with number of all samples (42), but in other rows all percentages were calculated in comparison with total number of that column.
* S. aureus Enterotoxins A and B (SEA and SEB)
Discussion

The experimental results of 42 cheese samples revealed that about 72% of them were contaminated with Staphylococcus spp. and 50% of samples were contained S. aureus gene. In addition, three samples were found to be highly contaminated with S. aureus. About 42.84% of isolates were producer of S. aureus enterotoxin. These results indicate high contamination rate of koozeh cheese samples to S. aureus, which can threaten health of local consumers. Cheese is one of the main foods consumed in Iran. Food industry professionals should consider microbial contamination. Cheeses produced in the villages traditionally and regardless of hygienic, can be an important source of pathogens for public health and it can play an important role for distribution of resistant infections to treatment. Several sources can contaminate cheese with S. aureus. In a previous study, researchers collected a total of 370 milk samples belong cows with clinical mastitis and subclinical in East and West Azarbaijan in Iran. They found that 58 of their local cheeses were contaminated with S. aureus (21). Koozeh cheese has a special production process. It recommended traditionally, after formal cheese production with local starter, it have to bury for a long

Figure 1 - Typical amplicon of S. aureus isolates isolated from Koozeh cheese samples. M: 100 bp standard marker. Line 6: S. aureus isolate XX (812 bp); lines 8: S. aureus ATCC® 25923TM (positive control), line 9: Negative control (distilled water); line 10: S. aureus isolate YY (530 bp).

Figure 2 - Molecular typing of S. aureus isolates from Koozeh cheese using RAPD PCR typing method. Including 21 isolates from cheese and one standard strain of S. aureus ATCC® 25922™. No clonal similarity or relationship was observed in isolates. (generated by Bionumerics gelcomare software).
time and after processing, has to mixed with local vegetables for final production. It can be noted that the pH decrease is accompanied by an increase of age in cheese. It will lead to a reduce in antimicrobial substances production by means of lactic acid bacteria, especially bacteriocins (22). With the passage of time and increase the ripening of traditional cheese, \textit{S. aureus} count will be decreased (23). However, in contrary, in our study cheeses had more than a year old and high-count number of \textit{S. aureus}. They were not resistant to any antibiotics and health treating bacteria. However, Because of the role of food in distribution of resistance, it should be considered for further processing and reducing possible contaminations with \textit{S. aureus}. Nowadays, emergence of MRSA in food product is one of the most treating health problems (8). In addition, All isolates were genetically divers and there was no cluster including two or more member. This finding indicates diversity and presence of different source of contamination. Molecular typing will help us for source case tracing and finding possible outbreaks. However, in this case, all isolates were diverse and it shows these cheeses were contaminated with different sources of \textit{S. aureus} (Figure 2).

A previous study showed proper sanitary conditions, can be prevented the growth of \textit{S. aureus} in food products, especially in Cheese (24). In addition, cheese obtained from raw milk compared to cheese produced from pasteurized milk, had higher containing of \textit{S. aureus} (24). According to a study, 75\% of raw cow milks and 10\% of cheeses were contaminated to \textit{S. aureus} (10). It shows possible difference in contamination. Because Koozeh Cheese has long process and manipulation, it increases the risk of contamination with \textit{S. aureus}. Ikeda evaluated cheese factories in Hokkaido. In their study, 3.6 - 9.2 \% of total cheese samples and 13 - 20 \% of mozzarella cheeses were contaminated to \textit{S. aureus}. In Most of cases, bacteria had 20,000 (CFU/g) count. All isolated samples were enterotoxin producer (25). However, in our study about 50\% of isolates were enterotoxins producer. In addition, these reports had lower rate of contamination with \textit{S. aureus} compared to our study with 72\% contamination. Several studies have shown a series of pathogenic toxins, especially Staphylococcus enterotoxin A and B in a sample of milk and cheese may be responsible for the development of nausea, abdominal cramps and diarrhea (26-29). Therefore, rate of enterotoxin producer isolates is important and reducing contamination and controlling these local products can prevent their side effects and poisoning or contaminations.

**Conclusion**

Traditional cheese in various regions has different process of production and even has a variable flora. For this reason, the prevalence of \textit{S. aureus} in traditional cheese is different. It should be mentioned that with the passage of time and increase the ripening of traditional cheese, \textit{S. aureus} count has to be decreased. However, these results indicate high level of microbial contamination in Koozeh cheese. About half of isolates were enterotoxin producer and had high diversity and no clonal relationship. Long Processing and manipulation can play an important role in contamination. Improvement in hygiene, training local manufacturers of Koozeh cheese, control of products for possible contamination and developing new protocols is needed to decrement of \textit{S. aureus} contamination in Koozeh Cheese products.

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Conflicts of Interest
All Authors declare no conflicts of interest.

Ethical Statement
Since this research has not done on laboratory animals and no human participating, any Ethical approve is required.

Conflict of Interest
None to declare

References


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