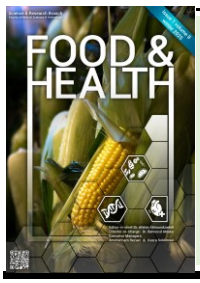

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Isolation and Identification of Coagulase Positive Staphylococcus aureus in Cow Raw Milk in Tehran by Culture and Real-Time PCR Methods

Saba Mohammadzadeh[1], Jalal Shayegh*[1]

[1] Department of Veterinary Medicine, Faculty of Veterinary and Agriculture, Shabestar branch, Islamic Azad University, Shabestar, Iran.

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ABSTRACT

Coagulase-positive *Staphylococcus aureus* (*S. aureus*) is one of the main causes of contamination of raw milk and dairy products, which can lead to food poisoning in humans. The rapid and accurate identification of this bacterium is of great importance. The aim of this study was to investigate the contamination of raw milk from Tehran cattle farms with *S. aureus* using culture and real-time PCR methods. For this study, 60 raw milk samples were collected from different cattle farms in Tehran. The samples were first analyzed using the culture method on Baired-Parker agar medium. The colonies with black centers and light areas were used for the coagulase test with rabbit plasma. The real-time PCR technique and the transcriptional regulator gene (*Sa0836*) specifically developed for *S. aureus* were then used to confirm the results. Of the 60 raw milk samples, 40 samples (66.66%) were identified as *Staphylococcus* in the culture medium. Of these, 32 samples (53.33%) were positive in the coagulase test. The results of the molecular tests also showed that *S. aureus* infection was confirmed in 27 samples (45%). The results of this study show that the contamination of raw milk in Tehran is significant and that the use of culture methods as a gold standard together with molecular techniques such as real-time PCR can help to increase the speed and accuracy of detection of *S. aureus* in raw milk. This approach can help to improve the quality and safety of dairy products and maintain public health.

1. Introduction

Milk is one of the most important foods in the human diet and plays an important role in the health and growth of people, especially children. Also, milk is a rich source of proteins, calcium, vitamins, and minerals that are essential for the growth and development of the body (1). Because milk is a suitable place for bacteria to grow and multiply, it can contain pathogenic bacteria such as coagulase-positive Staphylococcus aureus (S. aureus), which can endanger the health of consumers if consumed (2, 3).

Staphylococcus aureus is the most clinically important coagulase-positive species (4). Coagulase is an enzyme that can convert fibrinogen (soluble) directly into fibrin (insoluble) and cause the formation of blood clots. The enzyme coagulase helps S. aureus to escape the host's immune system by forming a fibrin network around the bacterial cells and limiting the spread of infection (5). Although the presence of the coagulase enzyme alone is not necessary for the pathogenesis of S. aureus, it is considered a good indicator for the identification of potentially pathogenic strains of S. aureus (6). Various studies have shown that infections caused by coagulase-negative staphylococci are often less severe and easy to treat compared to coagulase-positive staphylococci (7- 9).

Coagulase-positive S. aureus is one of the most important foodborne pathogens that can cause food poisoning in humans (10). This bacterium is commonly present in the raw milk of cows and there is a possibility of contamination of dairy products if the animal is affected by mastitis, or poor hygiene during the milking, storage, and transportation phases (11, 12). Therefore, the accurate identification and isolation of this bacterium in raw milk is of great importance to control and prevent the occurrence of poisoning by this bacterium in consumers of dairy products.

Bacterial culture methods are considered the gold standard for the identification of S. aureus, but due to its time-consuming nature and the need for laboratory expertise, molecular methods such as real-time PCR have been used in recent years for the rapid and accurate detection of this bacterium in raw milk. This method, which uses specific

*Corresponding author: Department of Veterinary Medicine, Faculty of Veterinary and Agriculture, Shabestar branch, Islamic Azad University, Shabestar, Iran.

E-mail address: Jalalshayegh@gmail.com (Jalal Shayegh).

primers and probes, can identify and confirm the presence of S. aureus in raw milk samples in a very short time (13-16).

According to the latest report of the Iranian Statistical Center, Tehran province produced the largest amount of milk in our country. The analysis of statistical results in 2017 showed that 5 million tons of milk were produced by 14,018 active dairy farms, with the highest production in Tehran Province at 1,106,000 tons. Therefore, the aim of this study is to isolate and identify coagulase-positive S. aureus bacteria in raw milk samples from Tehran cattle farms using the culture method and to verify the results obtained using the real-time PCR technique. The results of this study can be used to improve the health and safety of dairy products and prevent the occurrence of food poisoning caused by S. aureus.

2. Materials & methods

2.1. Sample collection

This study was a descriptive-analytical study and was conducted as a cross-sectional study. From January to March 2024, 60 raw milk samples were randomly collected from eight dairy farms in Tehran. The method of milk collection was that the milk in the milk container was first mixed thoroughly with the help of an agitator until it was uniform, and then 250 ml of milk was collected separately and randomly by special sterile containers. All samples were taken to the microbiology laboratory as soon as possible under aseptic conditions and on ice and analysed immediately.

2.2. Isolation and identification of S. aureus

2.2.1. Cultivation of samples

The samples were plated on Baird-Parker agar (Merck, Germany) enriched with egg yolk and tellurite emulsion (1%). For this purpose, 1 ml was taken from the milk samples under completely sterile and aseptic conditions and close to the flame and added to the tube containing 9 milliliters of sterile Ringer's solution and mixed well. The resulting mixture was used as the starting suspension for the preparation of the subsequent decimal dilutions. After making the required dilutions, 0.1 ml of the prepared dilutions were inoculated onto the Baird-Parker agar culture medium and spread completely over the surface of the plate using an L-shaped Pasteur pipette. The plates were then placed in an incubator at 37ºC for 48 h. At the end of the incubation period, colonies with a black or gray appearance surrounded by opaque zone, convex center with a diameter of 1-1.5 mm were marked. To confirm the diagnosis, the coagulase test with rabbit plasma, the catalase test, the oxidase test and the Gram stain were performed. The colonies that had the same shape and were positive for coagulase and catalase and negative for oxidase were subjected to molecular testing.

2.2.2. DNA extraction and real-time PCR

DNA extraction of the confirmed colonies from the culture method was performed using the High Pure PCR Template Kit (Roche, Switzerland) and according to the manufacturer's instructions. The primer and probe sequence from Table 1 synthesized by Sinaclon, Iran, was used to amplify the putative transcriptional regulator gene of S. aureus (Sa0836).

Real-time PCR was performed using the RealQ Plus 2x Master Mix for Probe Kit (Ampliqon). Each reaction mix (25 µL) was prepared as follows: 12.5 µL PCR Master Mix, 0.4 µL TaKaRa Ex Taq HS (5U µL-1), 1 µL of each primer, 0.25 µL Prob (10 µM), 5 µL DNA template, and 5.25 µL sterile deionized water. Amplification and detection were performed using the Rotor-Gene Q (Qiagen). Real-time PCR amplification was performed with the program according to the Goto et al, 2007 study (16). For negative controls, template DNA was replaced with sterile water. S. aureus ATCC25923 was used as positive control.

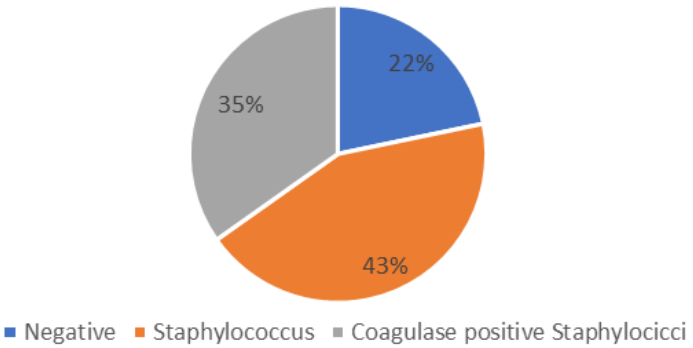
Table.1. Primer and probe sequences.

Primers and probe name	Oligonucleotide sequences (5′ to3′)	Reference	PCR product size (bp)
Sa0836F	TCG-AAA-TTAAAT-GTT-GTC-GTG-TCT -TC	(16)	285 bp
Sa0836R	TCA-TTT-TTGACA TGR-AGA-GAA ACA-TC		
Probe	FAM-TCG-CGA-CAT TCA-TTA-TGC-CCA AAT-TTTTAA -MGB		

3. Results

3.1. Frequency of contamination in culture and molecular methods

In general, the results of the culture of the samples showed that out of 60 raw milk samples, 40 samples (66.66%) were detected as Staphylococcus on the Baird-Parker agar medium and of these 32 samples (53.33%) were positive in coagulase test (Graph 1 and Table 2).



Graph 1. The number and frequency of Staphylococcus infected samples.

Table 2. Number and frequency of coagulase positive S. aureus-infected samples in microbial and real-time PCR tests.

Test type	Number of sample	Number and percentage of positive cases
Microbial culture	60	32 (53.33%)
Molecular		27 (45%)

According to the results of the complementary molecular test, 27 samples (45%) were found to be infected with S. aureus (Table 2). The amplification curve of the real-time PCR is shown in Figure 1.

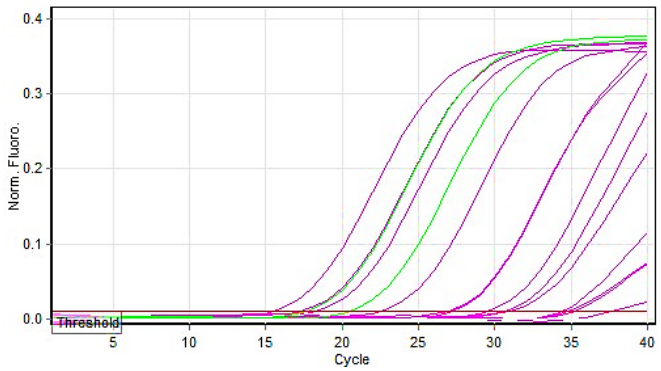


Figure 1. Amplification curve of Sa0836 in real-time PCR.

4. Discussion

Considering the importance of milk and dairy products for human nutrition and the health of society, it is very important to pay attention to their health and the absence of contamination with pathogenic microorganisms (17, 18). Therefore, we decided to investigate the prevalence of coagulase-positive S. aureus, one of the most important causative agents of mastitis in cows, in raw milk samples from Tehran cattle farms using culture and molecular methods (real-time PCR). According to the results of the present study, out of a total of 60 raw milk samples from Tehran cattle farms, 40 samples (66.66%) were infected with Staphylococcus using the culture method, and of these, 32 samples (53.33%) were coagulase positive. Considering that the use of culture methods is considered the gold standard for the identification of S. aureus, the use of molecular methods such as real-time PCR can increase the speed and accuracy of the detection of this bacterium in raw milk alongside the culture method. In this study, we used a transcriptional regulator gene (Sa0836) that is specific and universal for S. aureus (16, 19). The sequence of this gene was determined in 12 strains of S. aureus for the design of primers and probes (16). According to the results of the complementary molecular tests in 32 positive samples in the culture method, S. aureus infection was confirmed in 27 samples (45%). This finding shows the significant prevalence of this bacteria in raw milk. This result is similar to reports from other studies conducted worldwide and in Iran, because, in other studies, the results showed that milk samples contain a certain percentage of S. aureus contamination.

In studies conducted in Iran and other countries, including the cities of Tehran, Tabriz, Zanjan, the Brazilian state of Sao Paulo, and Shanghai, the contamination rate of raw milk with staphylococci was in the range of 4-25.6%. Fooladi et al. (2010) analysed 100 samples of dairy products, including milk, cream, and cheese, in four regions of Tehran for the presence of S. aureus. The results showed that a total of 32% of the dairy products were contaminated, 4% of which were in milk (20). In the study by Hassani et al. (2014) in Iran, 43% of dairy samples were infected with S. aureus, of which 22% were detected in milk samples (21). Saadat et al. (2014) identified S. aureus in 27% of milk and cheese samples in Tabriz (22). In the study by Haghi et al. (2019), a total of 82 samples of unpasteurized cow's milk were collected from cattle farms in different rural areas of Zanjan and the prevalence of S. aureus was investigated by culture and molecular methods. The results of their study showed that 21 samples (25.6%) were infected with S. aureus by culture method (23). Shariatifar et al (2023) investigated the prevalence and antibiotic resistance of coagulase-positive strains of S. aureus in raw milk from cattle farms in Mashhad. The results of their study showed that out of 250 milk samples, 18.4% were infected with S. aureus (24). Fagundes et al. (2010) investigated the presence of S. aureus in raw milk from dairy farms in the state of São Paulo, Brazil, and showed that out of 208 samples of cow's milk with subclinical mastitis and 37 samples of bulk milk, 14 samples (7. 6%) and 4 samples (10.8%) were infected with S. aureus, respectively (25).

In a study in Shanghai in 2015, Song et al showed that 50 samples (20.2%) of 248 raw milk samples contained S. aureus (26). In general, this difference in prevalence rate may be caused by the different sampling seasons, sampling methods, geographical areas, and isolation and identification methods.

Contamination of raw milk with S. aureus may be largely due to subclinical mastitis, which is also reported in our country, in addition to the cases already mentioned (27- 29). Since the diagnosis of mastitis in the clinic is difficult, the risk of contamination of raw milk with the pathogen is higher in this case and it is necessary to use rapid and accurate diagnostic methods to control contamination and improve food safety (16, 30). Considering that the real-time PCR method was used to identify virulence genes in Staphylococcus, to our knowledge, this is the first study conducted in Iran using the Sa0836 gene.

In general, the results of this study show that the contamination of raw milk from Tehran cattle farms with coagulase-positive S. aureus is significant and that the use of rapid and accurate diagnostic methods together with hygiene measures during the production and distribution chain can reduce this contamination and should effectively prevent the occurrence of food poisoning.

5. Conclusion

The results of the present study showed that out of 60 cultured raw milk samples, 40 samples (66.66%) were infected

with Staphylococcus, and of these, 32 samples (53.33%) were positive for the coagulase test. By performing molecular tests, contamination was confirmed in 27 samples (45%). These results show that, firstly, contamination of raw milk from Tehran cow farms with coagulase-positive S. aureus is significant, which can lead to food poisoning in humans. Second, the combination of culture methods and real-time PCR can be used as an effective approach to identify and control the contamination of raw milk with coagulase-positive S. aureus. Thirdly, the result of this study makes it necessary to control the contamination of raw milk and to inform society about the dangers of raw milk consumption.

6. Acknowledgement

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7. Conflict of interest

None declared.

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