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Prevalence of Methicillin-Resistant Staphylococcus Aureus and Antibiotic Residue in Pasteurized and Raw Farm Milk in Tehran

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ABSTRACT

Background: There is a global threat of methicillin-resistant *Staphylococcus aureus (S. aureus or* MRSA), which has been regarded as a priority pathogen by the world health organization (WHO). Livestock and its products are the sources of MRSA which can often occur in poor breeding conditions. The present study aims to investigate the prevalence of MRSA and the rate of antibiotic residue in pasteurized and raw farm milk. **Methods:** This was a cross-sectional study conducted from April to July 2020. 250 samples (200 samples of raw milk in farms around Tehran and 50 samples of pasteurized milk) were cultured to evaluate the occurrence of *S. aureus* and its antimicrobial susceptibility profile to 7 antimicrobial panels. Hansen Kit was used to monitor antibiotic residue in milk. **Results:** 63 *S. aureus* isolates (25.2%) were detected from 250 milk samples. Among 200 raw milk samples, 48 (24%) *S. aureus* isolates were detected and no strain of *S. aureus* was isolated from pasteurized milk. The highest rates of resistance belonged to ampicillin (95.8%), amoxicillin/clavulanic acid (87.5%), tetracycline (50%), and cefoxitin (45.8%). Moreover, 43 (17.2%) out of 250 milk samples had antibiotic residue in the antibiotic residue test using Danish Hansen kit. **Conclusion**: The present study indicates a high prevalence of subclinical *S. aureus* in dairy herds in Tehran, Iran. The milk contaminated with *S. aureus* and MRSA, posed a risk to public health owing to the presence of a phenotype resistant to very common antibiotics.

Keywords: *Milk, Staphylococcus aureus, Methicillin, Anti-bacterial agents*

Introduction

Dairy products like milk are of great importance for human physical and cognitive growth owing to their nutrients for healthy growth (Pereira, 2014). However, the products may contain pathogenic microorganisms which affects their transmission to humans (Dhanashekar *et al.*,

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2012, Fard *et al.*, 2019). *Staphylococcus aureus* (*S. aureus*) is a pathogenic bacterium isolated from milk. *The* bacterium can be detected in milk due to contamination during milking, or it may be found in the milk obtained from cows affected by clinical subclinical mastitis caused by *S. aureus* or post-milk contamination (Grispoldi *et al.*, 2019, McMillan *et al.*, 2016, Mekonnen *et al.*, 2018). Subclinical mastitis caused by *S. aureus* and other pathogens seriously affects milk yield and composition of dairy cows (Gonçalves *et al.*, 2020).

The milk contaminated with *S. aureus* can be a source of serious infections and toxins (Dhanashekar *et al.*, 2012, Grispoldi *et al.*, 2021). Treatment of animals suffering from clinical and subclinical mastitis with antimicrobial drugs is usually performed to reduce economic and health consequences of mastitis in dairy cows. However, a large number of microorganisms resistant to some microorganisms due to improper use, can prevent the success of this treatment. In addition, antimicrobial-resistant *S. aureus* isolates and associated resistant genetic markers can be transmitted to humans through food chain, raising further public health concerns (Hammad *et al.*, 2012). Among all the resistance traits, methicillin-resistant *S. aureus* (MRSA) is clinically important, since MRSA isolates are resistant to the most common antimicrobial β-lactam group (Lee *et al.*, 2018, Soltan Dallal *et al.*, 2020).

Previous studies reported that 23.4 and 24.2 of raw cow milk samples in central Ethiopia and northwestern Ethiopia (Ayele *et al.*, 2017, Mekonnen *et al.*, 2018) were contaminated with *S. aureus*. High levels of resistance to commonly used antimicrobials were also reported. For example, Ayele et al. reported that 100% of the isolates were resistant to cefoxitin (methicillin), 98.5% to penicillin G, and 77.9% to streptomycin (Ayele *et al.*, 2017). However, Mckonnen *et al*, reported 86% rate of resistance to penicillin/ampicillin and 54% rate of resistance to tetracycline (Mekonnen *et al.*, 2018). Nevertheless, none of the isolates were resistant to methicillin. Understanding the predominant status of *S. aureus* in dairy products at different levels of production and their antimicrobial susceptibility is important for proper use of antimicrobial drugs in animals and humans and for developing possible alternatives in order to reduce the burden of antimicrobial strains. Therefore, the present study aims to investigate the occurrence of *S. aureus* and antimicrobial susceptibility profile isolated from milk and other dairy products in Tehran, Iran.

Materials and Methods

Study design and sample collection: A cross-sectional study was conducted from April to July 2020 to evaluate the occurrence of *S. aureus* and its antimicrobial susceptibility profile in raw milk and pasteurized milk. 250 samples (200 samples of raw milk in farms around Tehran and 50 samples of pasteurized milk available in shops) were cultured for *S. aureus* according to standard microbiological techniques. The working method was based on national standard of Iran No. 2406 (Iran national Standard, 2008).

Furthermore, 25 ml of raw cow milk mixture were collected from each sampling unit in the field in a sterile screwed bottle. 25 ml samples of pasteurized milk were collected from different brands available in shops in sterile bottles. Serious aseptic procedures were performed for collecting milk samples to prevent contamination. Bottles containing the samples were labeled and sent to Food Microbiology Laboratory of the School of Health and Tehran University of Medical Sciences, Iran, within 3-4 hours in containers with dry ice.

Isolation and detection of bacteria: First, 25 g/ml of each sample was separately added to 225 ml in Giolitti-Cantoni broth (Staphylococcus enrichment). After 24 hours of storage at 37 °C, a loop was inoculated on Baird-Parker agar. Black oil-blue halo colonies were considered suspect colonies, and thus differential tests, including catalase, coagulase, DNase test, mannitol salt agar test, and gram staining, were conducted. *S. aureus* ATCC25923 was also used as a reference strain at all the stages.

Antimicrobial susceptibility test: S. aureus isolates were tested for their susceptibility to

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common antimicrobial drugs in Mueller-Hinton agar (MHA) (Oxoid, Hampshire, UK) using Kirby-Bauer disk diffusion method based on the guidelines of Clinical & Laboratory Standards Institute (CLSI) (Cockerill, 2018, Jain *et al.*, 2008). Susceptibility of *S. aureus* isolates to 12 antibiotics (Mast Company, UK), including erythromycin (15 µg), amoxicillin (25 µg), tetracycline (30 µg), amoxicillin/clavulanic acid (30 µg), ceftriaxone (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), trimethoprim-sulfamethoxazole (25 µg), cefoxitin (30 µg), cephalothin (30 µg), ampicillin (10 µg), and oxacillin (1µ) was tested in the study.

The isolated *S. aureus* was inoculated in MHA, and its concentration was adjusted to the 0.5 McFarland standard after 4-5 hours at 37 °C. Using a sterile cotton swab, it spread evenly on the MHA medium, and the antimicrobial discs were placed on the plate 15 minutes after inoculating the bacterium. It was then incubated at 37 °C for 24 hours. The diameter of the inhibition zone was measured in millimeters using a caliper. Interpretation of susceptible, moderate, or resistant groups was determined according to the CLSI guidelines (Cockerill, 2018, Jain *et al.*, 2008). Phenotypic detection of MRSA strains was based on cefoxitin disc diffusion resistance (30 µg). The MRSA was detected in case of an inhibition zone halo diameter of greater than 22 mm (Cockerill, 2018).

Determination of antibiotic residue in milk: Copan Milk Kit was used (milk quality, Hansen Kit Company, Denmark) to determine the presence or absence of antibiotic residue in milk. Milk was added to the kit, and then was stored at 64°C for 3 hours (Movassegh, 2012). In the absence of antibiotics in milk, the indicator of microorganisms in milk or *Bacillus Stearothermophilus* feeds on nutrients and grows, changing the color of the medium from purple to yellow in the presence of bromocresol by fermenting lactose and producing acid. On the contrary, when antibiotics are present in milk, the antibiotic residue in milk prevents the growth of *Bacillus Stearothermophilus*, and the color of the medium does not change and remains purple.

Data analysis: Statistical analyses of the results were performed using SPSS (25 version). Descriptive statistics were used to show the prevalence of MRSA in raw and pasteurized milk and the percentage of antibiotic resistance to MRSA isolates.

Results

A total of 63 isolates (25.2%) were detected as staphylococci from 250 milk samples (including 200 raw milk samples and 50 pasteurized milk samples). Among the 200 raw milk samples, 48 (24%) were *S. aureus*, 6 (3%) , *Staphylococcus saprophyticus*, and 6 samples (3%) were *Staphylococcus epidermidis*. 3 isolates (6%) were obtained from pasteurized milk, including 2 *Staphylococcus epidermidis* isolates and 1 *Staphylococcus saprophyticus* isolate. No strain of *S. aureus* was isolated from pasteurized milk (**Figure 1**).

As shown in **Figure 2**, after determining the antimicrobial susceptibility profile, *S. aureus* isolates revealed 2.1–95.8% level of resistance pattern to the tested antibiotics. From 48 isolates of *S. aureus*, 46 (95.8%) isolates showed resistance to ampicillin, followed by resistance of 42 (87.5%) isolates to amoxicillin/clavulanic acid, 24 (50%) isolates to tetracycline, 22 (45.8%) isolates to cefoxitin, 22 (45.8 %) isolates to oxacillin, 12 (25.0%) isolates to erythromycin, 15 (31.25%) isolates to sulfamethoxazole-trimethoprim, 7 (14.58%) isolates to cephalothin, 3 (6.25%) isolates to gentamicin, 2 (4.1%) isolates to amoxicillin, 2 (4.1) isolates to chloramphenicol, and 1 (2.1%) isolate to ceftriaxone.

According to the results, from 250 milk samples, 43 samples (17.2%) were positive for antibiotic residue according to diagnostic kits. The results of antibiotic residue test were shown in **Figure 3**.

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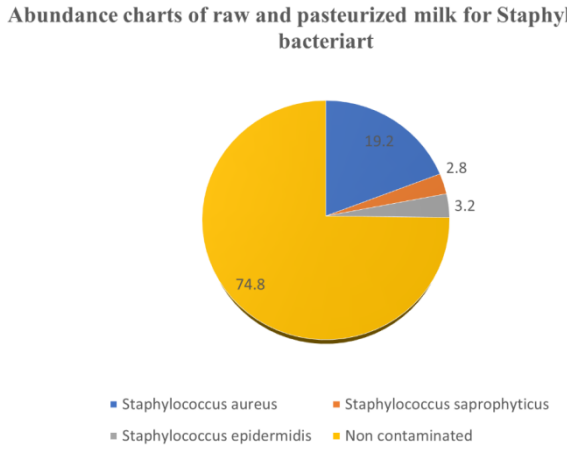

Figure 1. Abundance charts of raw and pasteurized milk for Staphylococcus bacteria.

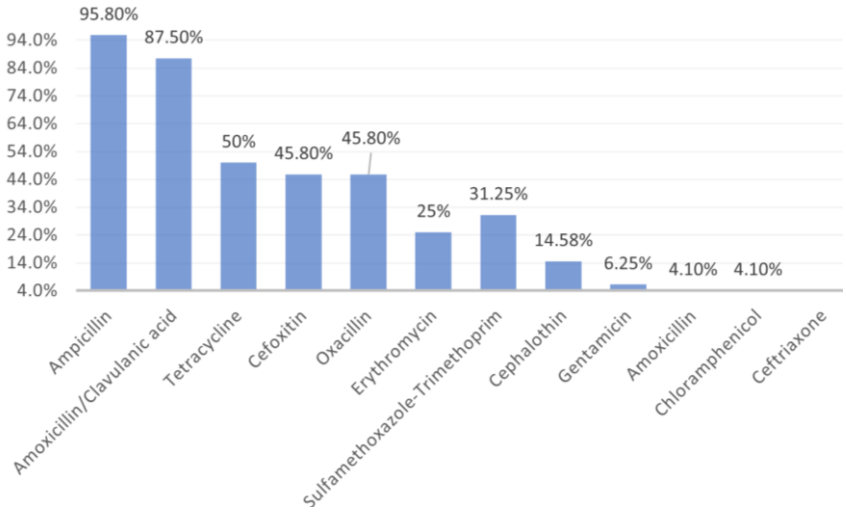

Figure 2. Antibiotic resistance percentages of bacteria for different antibiotic.

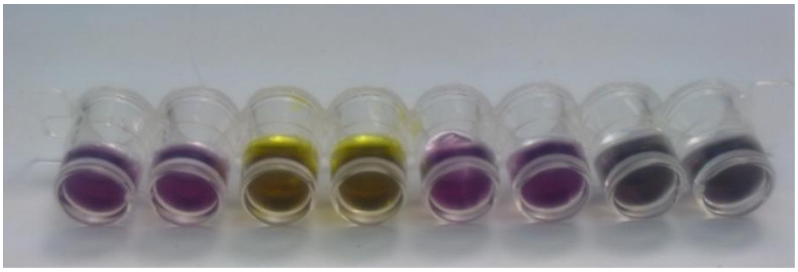

Figure 3. Evaluation of the presence or absence of antibiotic residue in milk using the Copan Milk Test [+ve: the presence of residue in milk (no color change), -V: the absence of residue in milk (color change from purple to yellow)].

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Discussion

The present cross-sectional study was conducted on dairy farms in Tehran. It demonstrated that among 200 raw milk samples, 48 (24%) samples were contaminated with *S. aureus* and 12 (6%) samples were contaminated with Coagulase-Negative Staphylococci (CoNS) which were considered important mastitis-causing bacteria.

S. aureus and Coagulase-Negative Staphylococci (CoNS) were the key bacteria causing mastitis in dairy cows (Djabri *et al.*, 2002, Xu *et al.*, 2015). The finding was consistent with previous studies in Nepal, indicating that *S. aureus* was a major bacterial pathogen causing clinical and supraclinical mastitis (Chandrasekaran *et al.*, 2014, Dhakal *et al.*, 2007). However, this study did not evaluate *S. aureus* isolates at genetic level, compared to previous studies.

Another study by Sori in Ethiopia revealed a 52% prevalence of *S. aureus* in cow's milk (Sori *et al.*, 2011). The prevalence of *S. aureus* in the current study was lower than other studies; because the reason might be that farmers preserved milk and other dairy products in a better situation in Tehran owing to their knowledge of hygienic practices.

In this study, significant resistance to microbes was observed in *S. aureus* isolates in milk, and high levels of resistance to ampicillin (95.8%) and amoxicillin+clavulanic acid (87.5%) were recorded. Previous studies in Ethiopia reported high levels of resistance similar to ampicillin and amoxicillin+clavulanic acid (Daka *et al.*, 2012, Mekonnen *et al.*, 2018). This was probably due to the frequent use of different β-lactams in research regions, contributing to selection of resistant strains. Similarly, high levels of resistance to tetracycline and sulfamethoxazole + trimethoprim in this and previous studies may be because of the overuse of these antimicrobials to treat and prevent various animal infections in the region (Ayele *et al.*, 2017).

In the present study, 45.8% of *S. aureus* isolates were phenotypically resistant to methicillin based on cefoxitin susceptibility, which was less than the percentage in the study by Ayele *et al.* where 100% of *S. aureus* isolates from milk value chain around Sebeta (Ethiopia) were resistant to cefoxitin (Ayele *et al.*, 2017).

S. aureus is an important food pathogen which can cause various diseases in humans, and its diagnosis in milk and other dairy products poses considerable risks to public health (Bintsis, 2017, Lemma *et al.*, 2021). Owing to healthcare costs, *S. aureus* has significant effects on general health, and the length of hospital stay increases when isolates are resistant to antimicrobial drugs, particularly MRSA, compared to susceptible strains (Zhen *et al.*, 2020). In the present study, resistance to 7 antimicrobials was detected in an isolate. Such significant levels of resistance in isolates indicated the need for strong regulation and prudent use of antimicrobial drugs.

Identification of antibiotic residue in Ilkhchi district in Tabriz using Copan Milk Kit indicated that 10% of 50 milk samples had antibiotic residue while 17.2% of the samples in the present study had antibiotic residue (Movassegh, 2012). The results of one study by Yamaki *et al.* in Spain indicated that only 1.7% of the 2686 samples regarding raw sheep milk contained antibiotic residue (Yamaki *et al.*, 2004). A study by Gidini *et al.* in Italy demonstrated that β-lactam antibiotic residue was found in 49% of cow's milk (Ghidini *et al.*, 2002). The difference between the results regarding previous studies and the present study can be attributed to infectious diseases (particularly mastitis). It can also be argued that failure to terminate the course in antibiotic-treated cows may be the main reason for antibiotic residue.

In developed countries, antibiotic residue is often found in cows treated with higher doses than allowed, while in developing countries, antibiotic residue is found in most samples due to non-compliance with the milking period. It is a major reason for the high contamination of food products with animal origin, particularly milk contamination with veterinary drugs in many regions of Iran, and this region compared to reports from developed countries. The abovementioned cases can be assessed using Copan and Delve tests, and the kits are designed to detect antibiotic residue at permissible level, since people regard milk and

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other dairy products as complete and healthy food products; hence, this attitude should be maintained and strengthened. As can be seen, milk quality and health control for antibiotic residue and the correct use of antibiotic is of great importance (Kurjogi *et al.*, 2019).

This study revealed a significant level of contamination of milk and dairy products with *S. aureus*, and most isolates were resistant to several drugs. In particular, the occurrence of MRSA in raw milk and dairy products indicated a serious threat to public health due to the widespread consumption of raw dairy products in the research region. The inconsistency between the phenotypic and genotypic diagnosis of MRSA in the present study requires further research to detect the genetic basis of such differences.

Conclusion

The present study demonstrated that raw milk with animal origin was contaminated with MRSA in farms, posing a serious health risk to consumers. An effective strategy is indispensable to ensure food safety and prevent the emergence or spread of MRSA through contamination of raw foods. The need to improve hygiene practices during food processing, distribution, and consumption of products should be further confirmed. It is recommended that dairy herds be screened for subclinical mastitis, and the animals be treated based on antibiotic susceptibility tests to reduce the prevalence of antibiotic resistance.

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Conflict of Interest

The authors declared no conflicts of interest.

Authors' contributions

Soltan Dallal MM conceived and designed the analysis. Agha Mirzaei HM participated in sample collection and performed all the experiments. Didar Z, Bakhtiari R, and Vahidi S analyzed data and interpreted the results. Soltan Dallal MM, Mohammadi MR, Mirbagheri SZ contributed wrote and revised the manuscript. Agha Mirzaei HM and Mohammadi MR prepared tables and figures. All the authors read and approved the final manuscript.

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