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Staphylococcus aureus Bacteria Resistant to Methicillin in Raw Milk

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The presence of antibiotic-resistant strains of *Staphylococcus aureus* (particularly methicillin-resistant strains) in food of animal origin is considered as a severe threat to human health due to numerous clinical complications. This study tended to determine the prevalence of methicillin-resistant *S. aureus* in samples of raw milk distributed in Tehran using antibiotic susceptibility testing methods. In the present study, 100 raw milk samples were taken from the centres of production and purchase of milk and its products in Tehran; the samples were evaluated by culture in terms of infection with coagulase-positive *S. aureus*. Finally, antibiotic resistance pattern of isolates was studied using disk diffusion agar. The average colony count was estimated. Raw milk cultures were estimated at 2×10^5-4×10^5 cfu/ml. Based on the results of culture, 36 samples of raw milk tested were icnfected with positive-coagulase *S. aureus*. The highest susceptibility was observed for ciprofloxacin and gentamicin (100%) and the highest resistance was observed to penicillin, tobramycin, oxacillin and ceftazidime. The results showed the prevalence of infection of raw milk

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with *S. aureus*. Moreover, prevalence of *S. aureus* resistant to a wide range of antibiotics, more importantly, methicillin resistant, was significant in the tested samples. Therefore, adherence to and control of sanitation in different stages of production, supply and consumption of milk can prevent human infection.

Keywords: Staphylococcus aureus; methicillin resistance; raw milk.

1. INTRODUCTION

Food-borne diseases are defined by the World Health Organization (WHO) as an infectious or poisonous disease caused by or thought to be caused by water or food. Foodborne diseases are a major public health problem from which millions of people worldwide suffer, and partly, lead to death or hospitalisation [1]. Food poisoning is a term used to express any illness, distress, or adverse effect which occurs after food intake [2]. *S. aureus* is one of the most common causes of bacterial food poisoning, which is considered as the second or third most important cause of these diseases. This bacterium is responsible for poisoning diseases such as toxic shock syndrome, Kawasaki syndrome and Staphylococcal food poisoning [3]. This bacterium is also one of the most common pathogens in infections of the population and hospital infections and can cause septicemia, endocarditis, osteomyelitis, abscess, pneumonia, wound infection, yellow ulcers, skin lesions and diseases caused by poisoning. *S. aureus* is also one of the major pathogens of clinical and sub-clinical mastitis in domestic dairy ruminants [4]. Food poisoning of this bacterium is caused by the presence of its enterotoxigenic strains in foods and its digestion. Poultry products, meat, eggs, as well as milk and dairy products are reported as common foods which can cause staphylococcal food poisoning [5].

S. aureus has several virulent factors to which pathogenicity and bacterial colonisation are attributed. Bacterial enterotoxins and toxic shock syndrome toxin (TSST-1) are important virulence factors of this bacterium [6]. This bacterium produces different enterotoxins. The isolates which have the sea to see gene and produce classical enterotoxins (A to E) account for 95% of staphylococcal food poisonings. Therefore, the presence of *S. aureus* in food can be a potential health hazard [7]. Milk and dairy products are foods which are exposed to infection with this bacterium. Infection may be transmitted through breast of the animal with mastitis or carriers. *S. aureus* enterotoxins are highly stable and are resistant to heat of pasteurisation and many proteolytic enzymes and can remain active in foods for a long time. The amount of enterotoxin required to cause symptoms of food poisoning is very low and can cause symptoms such as abdominal cramping, nausea, vomiting, and sometimes diarrhea. Therefore, sensitive methods are needed to detect staphylococcal enterotoxins even in small amounts [8]. Emergence and spread of antibiotic-resistant microbes has become a major concern over the last decade, and this increase in resistance has continued. The emergence of resistant strains against antibiotics in Staphylococci, due to the presence of antibiotic residues used in livestock, is a risk to humans and efforts to treat infections caused by these microorganisms cause resistance to most antibiotics, particularly methicillin [9].

Dehghani et al. [10] examined the prevalence and antibiotic resistance of *S. aureus* in raw and pasteurised milk. This descriptive and cross-sectional study was conducted in Sari, Iran, in the summer of 2014. Sherafati Chaleshtri et al. [11] determined antibiotic resistance pattern in coagulase-positive *S. aureus* strains isolated from ready to eat foods in Kashan. In this cross-sectional study, 384 samples (60 samples of salad, 40 samples of frozen vegetables, 120 samples of traditional ice cream, 90 samples of confectionery, 40 samples of hamburgers and 34 samples of kebabs) were randomly purchased from shops in Kashan and the prevalence *S. aureus* was examined by culture. Antibiotic resistance of isolates isolated by disk diffusion was investigated. Based on findings, 4 out of 384 samples (1.042%) had coagulase-positive *S. aureus*. Fazl Ara et al. [12] examined the presence of methicillin resistant gene (mecA) in *S. aureus* strains of food origin. Based on results of this study, 31 out of 146 food samples obtained from Ahvaz, Iran, were confirmed in terms of *S. aureus* in morphological and some biochemical properties. Of 31 positive strains, 7 strains were related to samosa (22.58%), 2 strains were related to Falafel (6.45%), one strain was related to cream (3.22%) and 27 strains were related to fresh milk of cows and buffaloes (87.09%).

Febler et al. [8] also showed that of 86 strains of *S. aureus* coagulase, 32 strains (37.2%) were MRSA, of which 6 were related to fresh chicken and 4 strains of chicken products andnd 11 strains of turkey meat. In 2013, Jackson et al. [9] Showed that of 63 strains of *S. aureus* coagulase isolated from beef, 4 strains (6.34%) had the mecA gene. Pexara et al. In 2013, conducted a study on the prevalence of MRSA in milk and dairy products, with the highest prevalence in Ethiopia, Africa (60.3%) and in Asian countries (28.3%). The lowest rates were reported from Korea and Japan. In the majority of European countries, the researchers report the incidence of MRSA from zero to low [13].

This study tends to isolate *S. aureus* from raw milk samples and investigate antibiotic resistance to methicillin by disc diffusion.

2. MATERIALS AND METHODS

2.1 Isolation of Bacteria

A total of 100 raw milk samples were taken randomly from production centers and purchase of milk and its products from different areas of Tehran in November and December 2017; 300 ml of each sample was transferred to laboratory of the Pegah Milk Factory in sterilised containers. Sampling was carried out according to the Institute of Standards and Industrial Research of Iran,(No. 3-6806). To count total microorganisms, raw milk samples were diluted; they were cultured on a plate count agar for 72 h at 30°C The samples were transferred to the laboratory according to the Institute of Standards and Industrial Research of Iran No. 6-6803; for enrichment of the samples, 5 g sample was first added to 25 ml sterile ringer serum and fixed for 15 min; then 1 ml sample mixed was added to 9 ml Giolitti-Cantonese medium (Merk, Germany). Giolitti-Cantonese medium contained 1% sterile Potassium Tellurite. This selected culture medium is enriched for Staphylococci, and growth of other bacterial species is stopped by Potassium Tellurite. This culture medium was incubated for 24 hr at 37°C. Then, the samples were taken with a pipette and transferred to Baird-Parker agar (Merk, Germany) and spread well over the culture medium using a curved glass rod. The plates were sealed so that the sample was completely absorbed by the medium and their surface was slightly dried; then, the plates were placed upside down in an oven at 37°C for 48 h. After 48 h, the plates were expelled from the oven; glossy black colonies with transparent halo were examined as suspected colonies in culture medium. Baird-Parker agar is a staph diagnostic medium (Institute of Standards and Industrial Research of Iran, No. 3-6806). After collecting the data, the results were presented in the form of frequency tables, charts and numerical indices. Chi-square test and Fisher's exact test were used to analyse the data. Data was analysed by SPSS 21 software.

2.2 Isolate Identification Tests by Phenotypic Methods

In order to determine the definitive identity and identify the isolates, experiments such as gram stain were used to observe bacterial morphology, catalase test, slide and tubular coagulase, growth on mannitol salt agar and microscopic observation. All gram positive, catalase positive, coagulase positive strains grown on salt agar mannitol were considered as *S. aureus* species. Materials and equipment required included hot staining kit, 3% oxygen dioxide, physiological serum, rabbit plasma, mannitol salt agar culture media, Dnase culture medium, hydrochloric acid, slide, and loop.

Gram stain: All gram-positive cocci were isolated by gram stain.

Catalase test: For this experiment, 3% hydrogen peroxide was first diluted to 1%. A small amount of bacterial colonies cultured in nutrient agar was removed by Pasteur pipette or any other appropriate means and placed on a slide. Then a drop of hydrogen peroxide was drained over it. Staphylococci were positive for catalase testing and cause air bubbles if added to 3% hydrogen peroxide and releasing oxygen. This test is important for differentiating staphylococci with streptococci grown in this medium [14].

Mannitol salt agar test: Manitol test can be used to differentiate *S. aureus* from other Staphylococcus species. To perform this test, the colonies produced in purification step were fed into Mannitol salt agar mediaum (Merk, Germany) made diagonally in the test tubes and surface culture was carried out. After incubation at 37°C for 24 h, if the bacteria were able to use mannitol sugar, pink color of the medium turned into yellow by producing acid.

Coagulase test using slide: To isolate *S. aureus* from other species, coagulase test is a very good tool which can be performed both in

tube and on slide. To carry out this test, human plasma can be recommended, while rabbit plasma (Sigma, UK) is widely used commercially. First, coagulase test was performed using slide. In this way, a colony of bacteria was completely dissolved in a physiological serum droplet; then, a rabbit plasma drop containing EDTA (Sigma, Germany) was added and mixed by rotating the slide to examine clot formation and positive result [14].

Tubular coagulase: Isolates which were negative in the slide technique were also tested by tubular method.

First, the citrate rabbit plasma was diluted to 1:5 (i.e., 1 cc plasma and 4 cc distilled water). Then, 0.5 ml diluted plasma was poured into the tubes and several colonies of bacteria were dissolved. Finally, tubes were incubated for 3-4 h at 35-37°C. After incubation time, if the clot was not visible and the result was negative, it was incubated at room temperature for 24 h. Because some strains, if placed at 35°C for a long time, produce fibrinolysin enzyme, which causes the clot to dissolve at incubation time; in the absence of clot, the result was considered negative. Positive and negative control strains were used to control plasma (Institute of Standards and Industrial Research of Iran, 2406).

2.3 Microscopic Observation

To observe *S. aureus* microorganisms under an optical microscope and to adapt their morphology to properties noted for this microorganism, black, glossy and convex colonies which preferably had a bright halo around them or white or yellow golden colonies formed in agar were transferred on a clean slide containing a sterile physiological serum droplet. After stabilising, gram stain was done. The slide was observed under a microscope with a lens of 100; germ-positive cocci-shaped bacteria which were arranged in the form of cluster were observed [14].

2.4 Determining Antibiotic Susceptibility Pattern by Disc Diffusion Agar

For antibiotic sensitivity test, 0.5 McFarland standard was made from bacteria. To make 0.5 McFarland (1.5×10^8 ml), 0.5 ml 0.048 M Barium chloride (BaCl2) was added to 99.5 ml 0.18 M Sulfuric acid. In addition, the standard is stable in dark and room temperature for 6 months. It was used as a standard cell suspension for antibiotic sensitivity. Standard correct turbidity density was determined using a 625 nm spectrophotometer. OD of 0.5 McFarland is 0.08-0.13 at this wavelength.

The Muller Hinton Agar (Merk, Germany) was made according to CLSI instruction that to perform a disk diffusion agar test. For this purpose, the medium was spread in 12 cm plates to a depth of 4 cm and incubated at 35°C for 24 h, after sealing the medium in plates. From 18-24 h culture of bacteria grown in nutrient agar, a suspension was made with turbidity equivalent to 0.5 McFarland. Then, the suspension was sterilised by a sterile swab on a Muller-Hinton Agar medium in three different directions; after a few minutes, antibiotic discs (MAST, UK) were placed 22 mm apart and 16 mm from the plate wall on the medium. Then, it was incubated at 35°C; the non-growth halo diameter was read with a ruler for all antibiotics. There are standard tables in which diameters are obvious for any bacterium and any antibiotic in the absence of growth. Then, the results were matched with the tables (CLSI, 2006). The standard strain of Enterococcus faecalis ATCC 29212 and Trimethoprim/sulfamethoxazole disc were used for qualitative control of the Muller-Hinton Agar and the standard strain of *S. aureus* ATCC 25923 was used to control antibiotic sensitivity testing.The antibiotics used in this project are based on Table 1.

Table 1. Antibiotics used in disk diffusion

Antibiotic	Value
Ciprofloxacin	5 µg
Oxacillin	1 µg
Gentamicin	10 µg
Tetracycline	30 µg
Erythromycin	15 µg
Chloramphenicol	30 µg
Cotrimoxazole	5 µg
Rifampinsin	5 µg
Vancomycin	30 µg
Penicillin	10 µg
Tobramycin	10 µg
Ceftazidime	30 µg
Methicillin	5 µg

3. RESULTS

3.1 Total Count of Microorganisms in Raw Milk

Table 2 compares colony count per milliliter of raw milk in this study with standard values of colony count in raw milk culture medium,

including the Institute of Standards and Industrial Research of Iran (2406), the FDA standard, the EEC standard, the CFIA standard, and the USDA standard. The table shows that the raw milk used in this study is classified as Grade 2 in terms of infection. The average number of colonies counted in raw milk cultures was determined by ocular counting per ml of milk in the range of 2×10^5-4×10^5 ml/cfu.

3.2 Identification of *S. aureus* by Phenotypic Methods

Baird-Parker agar is diagnostic medium of staphylococci. Glossy black colonies with transparent halo were investigated as suspected colonies in culture medium. Fig. 1 shows a number of positive plates in terms of staphylococcus growth.

Of 100 samples of raw milk, 43 samples were positive in Baird-Parker agar and black colonies were formed in the medium (Fig. 2).

To isolate *S. aureus* from other species, coagulase test is a very good tool which can be used both in tube and on slide. This study used slide and tubular coagulase for isolation of *S. aureus* strains. Clotting was considered as positive result for coagulase test. Fig. 3 shows slide and tubular coagulase test.

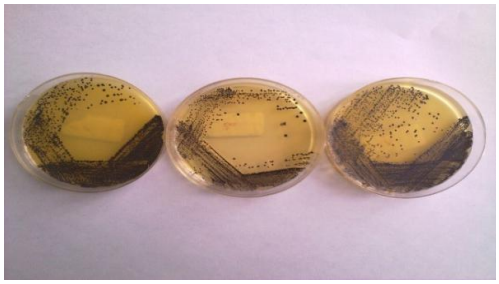

Fig. 1. Formation of black colonies in Baird-Parker agar

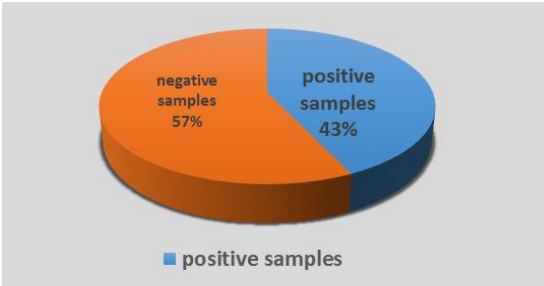

Fig. 2. Prevalence of *Staphylococcus* strains in raw milk

Table 1. Acceptable quality of raw milk for total number of microorganisms according to national and international standards (ml/cfu)

Standards quality degree	Institute of standards and industrial research of Iran	USDA	CFIA	EEC	FDF	Current study
Excellent	3×10^4	2×10^4	-	2×10^4	3×10^4	
Grade 1	3×10^4-10^5	-	-	2×10^4-10^5	3×10^4-10^5	-
Grade 2	10^5- 5×10^5	10^5<	-	10^5<	10^5-5×10^5	2×10^5-4×10^5
Grade 3	5×10^5-10^6	-	-	-	5×10^5-10^6	-
Acceptable maximum	-	10^5	5×10^5	10^5	-	-

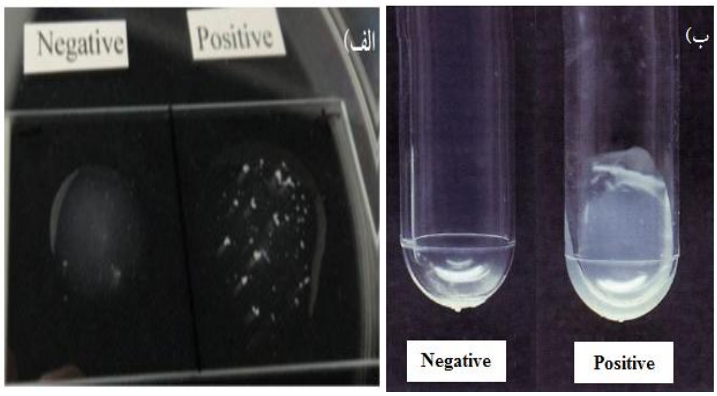

Fig. 3. Coagulase test, a) Slide coagulase test, b) Tubular coagulase test

According to coagulase test, 36 cases were coagulase positive and were infected with coagulase positive *S. aureus*. Of 100 raw milk samples collected from Tehran, 36 samples (36%) were infected with *S. aureus* and 64 (64%) confirmed the absence of infection.

According to available standards, the number of potential organisms required by *S. aureus* bacteria per milliliter milk for human disease is in the range of 10^6-10^9. Many studies have been conducted on infection of dairy products, indicating the infection of raw milk and its products produced traditionally versus industrially [1].

3.3 Antibiotic Susceptibility Pattern of *S. aureus* Strains

By assessing the lack of growth on antibiotic disks (Fig. 4) and comparing with the latest CLSI (Clinical and Laboratory Standards Institute), sensitivity of methicillin-resistant *S. aureus* strains to other antibiotics was investigated. Based on disc diffusion agar, 24 out of 36 isolates from raw milk samples (66.67%) were resistant to methicillin.

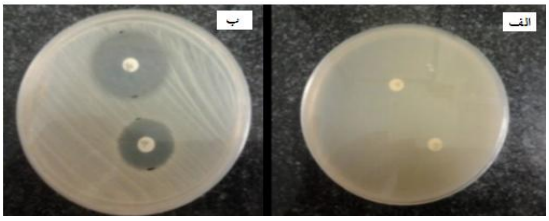

Fig. 4. Disc diffusion with antibiotic sensitivity discs. A) Antibiotic susceptibility, B) Antibiotic resistance

Table 3 shows the frequency and percentage of resistance to various antibiotics in 36 confirmed strains at culture of raw milk samples.

4. DISCUSSION

In this study, the highest antibiotic resistance in MRSA strains was observed to penicillin, tobramycin and Ceftazidime; 36 strains (100%) were resistant to these antibiotics. Moreover, 31 strains (86.11%) were resistant to oxacillin, followed by methicillin (66.7%), vancomycin (38.9%), erythromycin (16.7%), rifampicin (13.9%), chloramphenicol (8.3%) and cotrimoxazole (5.6%). The lowest resistance was observed to ciprofloxacin and gentamicin (Table 4). Many studies have been done on antibiotic susceptibility of *S. aureus*.

Table 2. Frequency and percentage of resistance of *S. aureus* strains to different antibiotics

Antibiotic resistance	N	%
Ciprofloxacin	0	0
Oxacillin	31	86.11
Gentamicin	0	0
Tetracycline	16	44.44
Erythromycin	6	16.67
Chloramphenicol	3	8.3
Cotrimoxazole	2	5.56
Rifampicin	5	13.89
Vancomycin	14	38.89
Penicillin	36	100
Tobramycin	36	100
Ceftazidime	36	100
Methicillin	24	66.67

Table 3. Comparison of frequency of antibiotic susceptibility of methicillin-resistant and -susceptible *S. aureus* strains against common antibiotics

Antibiotic	Antibiotic susceptibility pattern	
	Resistant (%)	Sensitive (%)
Ciprofloxacin	0	100
Oxaziline	86.11	13.89
Gentamicin	0	100
Tetracycline	44.44	55.56
Erythromycin	16.67	83.33
Chloramphenicol	8.3	91.7
Cotrimoxazole	5.56	94.44
Rifampicin	13.89	86.11
Vancomycin	38.89	61.11
Penicillin	100	0
Tobramycin	100	0
Ceftazidime	100	0
Methicillin	66.67	33.33

Moon et al. [15] studied antibiogram and genetic diversity of *S. aureus* enterotoxin isolated from raw milk of cattle infected with breast infection from 140 dairy products in Korea in 1997 and 2004. Of 696 isolates of *S. aureus*, 7.2% were resistant to methicillin. Akineden et al. [1] collected and tested 181 goat cheese samples from the Hesse market in Germany and reported that 14 samples (17.7%) were infected with coagulase-positive staphylococci. Regarding the infection of raw milk in various studies, it can be claimed that factors such as infected feed, carriers, raw milk containers, water used to rinse these containers, mammary gland if they have mastitis, and infection of legs, muzzle and ulcers during milking can be sources of infection of raw milk with *S. aureus* [16,17].

Aragon-Alegro et al. [18] analysed 172 food samples including milk, soft cheese, hard cheese, ice cream, yoghurt and prepared foods such as sandwiches delivered in the Botucitu market, Brazil, and reported that 26 samples (15.1%) of the tested foods were coagulase positive *S. aureus*. In the study of Yousefi et al. [19] the highest antibiotic resistance of MRSA strains was observed to gentamycin (76.7%), rifampin (46.7%), doxycycline (36.7%), erythromycin (80%), and tetracycline (80%). Clearly, the uncontrolled and unmonitored use of antibiotics for treating or controlling human infection or as growth factors in animal food is one of the reasons for prevalence of antibiotic-resistant bacteria [20].

5. CONCLUSION

The increase in foodborne diseases and food poisoning as well as its economic and social problems has led to development of various studies in the field of healthy food production. Due to emergence of antibiotic-resistant *S. aureus* strains, the number of antibiotics available for treatment of these infections has decreased day by day. Some strains have resisted even against a large number of antimicrobial compounds, including antibiotics and antiseptics. Regarding the important role of dairy products in diet of families and consumption of traditional dairy products by rural people and their unwillingness to use pasteurised dairy products, this study was conducted to determine infection of raw milk with *S. aureus* bacteria and to determine antibiotic susceptibility pattern.

According to total count of microorganisms, the raw milk used in this study was classified as Grade 2 in terms of infection. Of 100 raw milk samples, 43 samples were positive in Baird-Parker agar in which black colonies were formed. According to coagulase test, 36 cases were coagulase positive and were infected with coagulase positive *S. aureus*. Based on disc diffusion agar, 24 out of 36 isolates (66.67%) of raw milk samples were resistant to methicillin. In this study, the highest antibiotic resistance in MRSA strains was observed to penicillin, tobramycin and ceftazidime and 36 isolates (100%) were resistant to them. Moreover, 31 strains (86.11%) were resistant to oxacillin. Adherence to health is essential in milking, collecting, transporting and maintaining milk; moreover, pasteurised. Since few and limited studies have been cobducted in this regard across the country, there is no comprehensive statistics and data available. Therefore, it is suggested to conduct more precise studies in research centera with more frequent supervision from the Ministry of Health on the food distributed in different geographic zones. By increasing the awareness of people about pasteurised dairy products and proper training of livestock breeders in adhering to hygienic precautions at the time of supplying dairy products and introducing people with diseases caused by dairy products, basic steps can be taken to reduce the incidence of these diseases. Finally, studies can be done on frequency of methicillin in different *S. aureus* strains by PCR and its antibiotic susceptibility.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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