

# Staphylococcus aureus Bacteria Resistant to Methicillin in Raw Milk

## ABSTRACT

The presence of antibiotic-resistant strains of *Staphylococcus aureus* (particularly methicillin-resistant strains) in food of animal origin is considered as a serious 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 centers 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  $2 \times 10^5$ - $4 \times 10^5$  cfu/ml. Based on the results of culture, 36 samples of raw milk tested were infected 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 prevalence of infection of raw milk 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 hospitalization [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 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 colonization 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 pasteurization 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. Emergence of resistant strains against antibiotics in Staphylococci, due to the presence of antibiotic residues used in livestock, is a

41 risk to humans and efforts to treat infections caused by these microorganisms cause resistance to  
42 most antibiotics, particularly methicillin [9].

43 Dehghani et al. [10] examined the prevalence and antibiotic resistance of *S. aureus* in raw and  
44 pasteurized milk. This descriptive and cross-sectional study was conducted in Sari, Iran, in the  
45 summer of 2014. Sherafati Chaleshtri et al. [11] determined antibiotic resistance pattern in coagulase-  
46 positive *S. aureus* strains isolated from ready to eat foods in Kashan. In this cross-sectional study,  
47 384 samples (60 samples of salad, 40 samples of frozen vegetables, 120 samples of traditional ice  
48 cream, 90 samples of confectionery, 40 samples of hamburgers and 34 samples of kebabs) were  
49 randomly purchased from shops in Kashan and the prevalence *S. aureus* was examined by culture.  
50 Antibiotic resistance of isolates isolated by disk diffusion was investigated. Based on findings, 4 out of  
51 384 samples (1.042%) had coagulase-positive *S. aureus*. Fazl Ara et al. [12] examined the presence  
52 of methicillin resistant gene (*mecA*) in *S. aureus* strains of food origin. Based on results of this study,  
53 31 out of 146 food samples obtained from Ahvaz, Iran, were confirmed in terms of *S. aureus* in  
54 morphological and some biochemical properties. Of 31 positive strains, 7 strains were related to  
55 samosa (22.58%), 2 strains were related to Falafel (6.45%), one strain was related to cream (3.22%)  
56 and 27 strains were related to fresh milk of cows and buffaloes (87.09%).

57 Febler et al. [8] also showed that of 86 strains of *S. aureus* coagulase, 32 strains (37.2%) were  
58 MRSA, of which 6 were related to fresh chicken and 4 strains of chicken products andnd 11 strains of  
59 turkey meat. In 2013, Jackson et al. [9] Showed that of 63 strains of *S. aureus* coagulase isolated  
60 from beef, 4 strains (6.34%) had the *mecA* gene. Pexara et al., In 2013, conducted a study on the  
61 prevalence of MRSA in milk and dairy products, with the highest prevalence in Ethiopia, Africa  
62 (60.3%) and in Asian countries (28.3%). The lowest rates were reported from Korea and Japan. In the  
63 majority of European countries, the researchers report the incidence of MRSA from zero to low [13].

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

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## 67 2. MATERIAL AND METHODS

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### 69 2.1 Isolation of Bacteria

70 A total of 100 raw milk samples were taken randomly from the centers of production and purchase of  
71 milk and its products from different areas of Tehran in November and December 2017; 300 ml of each  
72 sample was transferred to laboratory of the Pegah Milk Factory in sterilized containers. Sampling was  
73 carried out according to the The Institute of Standards. To count total microorganisms, raw milk  
74 samples were diluted; they were cultured on a plate count agar for 72 h at 30°C The samples were  
75 transferred to the laboratory according to the ISIRI No. 6-6803; for enrichment of the samples, 5 g  
76 sample was first added to 25 ml sterile ringer serum and fixed for 15 min; then 1 ml sample mixed was  
77 added to 9 ml Giolitti-Cantonese medium (Merk, Germany). Giolitti-Cantonese medium contained 1%  
78 sterile Potassium Tellurite. This selected culture medium is enriched for Staphylococci, and growth of  
79 other bacterial species is stopped by Potassium Tellurite. This culture medium was incubated for 24  
80 hr at 37°C. Then, the samples were taken with a pipette and transferred to Baird-Parker agar (Merk,  
81 Germany) and spread well over the culture medium using a curved glass rod. The plates were sealed  
82 so that the sample was completely absorbed by the medium and their surface was slightly dried; then,  
83 the plates were placed upside down in an oven at 37°C for 48 h. After 48 h, the plates were expelled  
84 from the oven; glossy black colonies with transparent halo were examined as suspected colonies in  
85 culture medium. Baird-Parker agar is a staph diagnostic medium (ISIRI, No. 3-6806). After collecting  
86 the data, the results were presented in the form of frequency tables, charts and numerical indices.  
87 Chi-square test and Fisher's exact test were used to analyze the data. Data was analyzed by SPSS  
88 21 software.

### 89 2.2 Isolate Identification Tests by Phenotypic Methods

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

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

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

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

108 **Coagulase test using slide:** To isolate *S. aureus* from other species, coagulase test is a very good  
109 tool which can be performed both in tube and on slide. To carry out this test, human plasma can be  
110 recommended, while rabbit plasma (Sigma, UK) is widely used commercially. First, coagulase test  
111 was performed using slide. In this way, a colony of bacteria was completely dissolved in a  
112 physiological serum droplet; then, a rabbit plasma drop containing EDTA (Sigma, Germany) was  
113 added and mixed by rotating the slide to examine clot formation and positive result [14].

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

116 First, the citrate rabbit plasma was diluted to 1:5 (i.e., 1 cc plasma and 4 cc distilled water). Then, 0.5  
117 ml diluted plasma was poured into the tubes and several colonies of bacteria were dissolved. Finally,  
118 tubes were incubated for 3-4 h at 35-37°C. After incubation time, if the clot was not visible and the  
119 result was negative, it was incubated at room temperature for 24 h. Because some strains, if placed at  
120 35°C for a long time, produce fibrinolysin enzyme, which causes the clot to dissolve at incubation  
121 time; in the absence of clot, the result was considered negative. Positive and negative control strains  
122 were used to control plasma (ISIRI, 2406).

### 123 **2.3 Microscopic Observation**

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

### 130 **2.4 Determining Antibiotic Susceptibility Pattern by Disc Diffusion Agar**

131 For antibiotic sensitivity test, 0.5 McFarland standard was made from bacteria. To make 0.5  
132 McFarland ( $1.5 \times 10^8$  ml), 0.5 ml 0.048 M Barium chloride (BaCl<sub>2</sub>) was added to 99.5 ml 0.18 M  
133 Sulfuric acid. In addition, the standard is stable in dark and room temperature for 6 months. It was  
134 used as a standard cell suspension for antibiotic sensitivity. Standard correct turbidity density was  
135 determined using a 625 nm spectrophotometer. OD of 0.5 McFarland is 0.08-0.13 at this wavelength.

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

147 the standard strain of *S. aureus* ATCC 25923 was used to control antibiotic sensitivity testing. The  
 148 antibiotics used in this project are based on Table 1.

149 **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  |
| Rifampicin      | 5 µg  |
| Vancomycin      | 30 µg |
| Penicillin      | 10 µg |
| Tobramycin      | 10 µg |
| Ceftazidime     | 30 µg |
| Methicillin     | 5 µg  |

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### 151 3. RESULTS

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#### 153 3.1 Total Count of Microorganisms in Raw Milk

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155 Table 2 compares colony count per milliliter of raw milk in this study with standard values of colony  
 156 count in raw milk culture medium, including the ISIRI (2406), the FDA standard, the EEC standard,  
 157 the CFIA standard, and the USDA standard. The table shows that the raw milk used in this study is  
 158 classified as Grade 2 in terms of infection. The average number of colonies counted in raw milk  
 159 cultures was determined by ocular counting per ml of milk in the range of  $2 \times 10^5$ - $4 \times 10^5$  ml/cfu.

160 **Table 2. Acceptable quality of raw milk for total number of microorganisms according to**  
 161 **national and international standards (ml/cfu)**

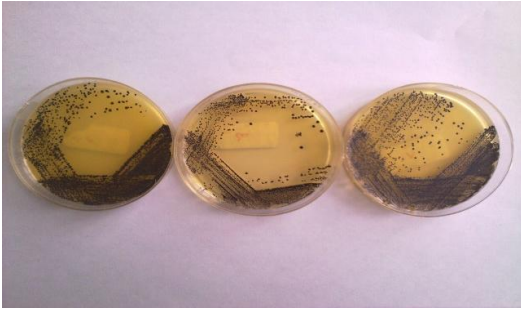
| Standards          | ISIRI                    | USDA            | CFIA            | EEC                      | FDF                      | Current study                     |
|--------------------|--------------------------|-----------------|-----------------|--------------------------|--------------------------|-----------------------------------|
| Quality degree     |                          |                 |                 |                          |                          |                                   |
| Excellent          | $3 \times 10^4$          | $2 \times 10^4$ | -               | $2 \times 10^4$          | $3 \times 10^4$          |                                   |
| Grade 1            | $3 \times 10^4$ - $10^5$ | -               | -               | $2 \times 10^4$ - $10^5$ | $3 \times 10^4$ - $10^5$ | -                                 |
| Grade 2            | $10^5$ - $5 \times 10^5$ | $10^5$ <        | -               | $10^5$ <                 | $10^5$ - $5 \times 10^5$ | $2 \times 10^5$ - $4 \times 10^5$ |
| Grade 3            | $5 \times 10^5$ - $10^6$ | -               | -               | -                        | $5 \times 10^5$ - $10^6$ | -                                 |
| Acceptable maximum | -                        | $10^5$          | $5 \times 10^5$ | $10^5$                   | -                        | -                                 |

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#### 163 3.2 Identification of *S. aureus* by Phenotypic Methods

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

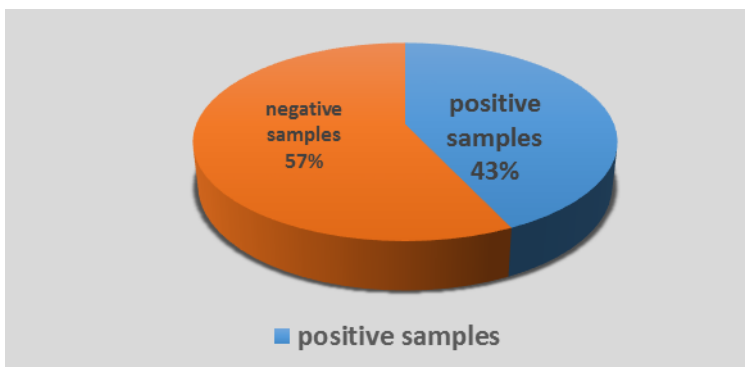


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169 **Fig. 1. Formation of black colonies in Baird-Parker agar**

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

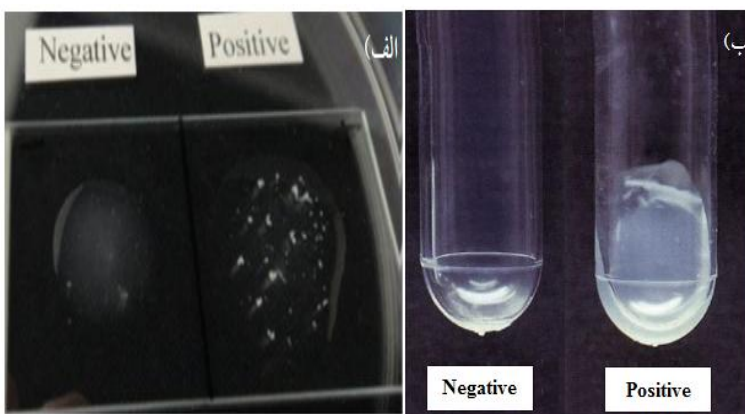


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174 **Fig. 2. prevalence of Staphylococcus strains in raw milk**

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



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

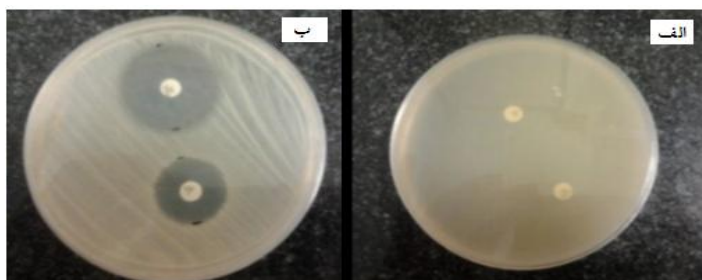
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183 According to coagulase test, 36 cases were coagulase positive and were infected with coagulase  
 184 positive *S. aureus*. Of 100 raw milk samples collected from Tehran, 36 samples (36%) were infected  
 185 with *S. aureus* and 64 (64%) confirmed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

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

### 190 3.3 Antibiotic Susceptibility Pattern of *S. aureus* Strains

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



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 197 **Fig. 4. Disc diffusion with antibiotic sensitivity discs. A) Antibiotic resistance, b) Antibiotic**  
 198 **susceptibility.**  
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200 Table 3 shows the frequency and percentage of resistance to various antibiotics in 36 confirmed  
 201 strains at culture of raw milk samples.

202 **Table 3. 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 |

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 204 **Table 4. Comparison of frequency of antibiotic susceptibility of methicillin-resistant and -**  
 205 **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 |

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#### 4. DISCUSSION

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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*.

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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].

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Aragon-Alegro et al. [18] analyzed 172 food samples including milk, soft cheese, hard cheese, ice cream, yogurt and prepared foods such as sandwiches delivered in the Botucutu 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].

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#### 5. CONCLUSION

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The increase in foodborne diseases and food poisoning as well as its economic and social problems have 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 pasteurized dairy products, this study was conducted to determine infection of raw milk with *S. aureus* bacteria and to determine antibiotic susceptibility pattern.

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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, pasteurized Due to the fact that there are very limited and fewer studies in the field of the transmission of infectious diseases caused by livestock products, there are no comprehensive statistics and data. Therefore, more detailed studies in research centers with more frequent monitoring by the Ministry of Health On foodstuffs supplied at various levels in the geographical areas of the community milk greatly reduces the number of people infected with foodborne infectious diseases. By increasing the awareness of people about pasteurized 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.

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