

## Prevalence of Methicillin-Resistant *Staphylococcus aureus* in Raw Milk and Dairy Products in Sarab by Culture and PCR Techniques

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**Abstract:** Recently Methicillin Resistant *Staphylococcus aureus* (MRSA) was isolated from dairy products in different countries. The aim of this study is to determine the prevalence rate of methicillin-resistant *Staphylococcus aureus* in raw milk, pasteurized milk, ice cream and traditional butter in Sarab by culture and PCR techniques. To do so, 200 samples of raw milk, pasteurized milk, ice cream and traditional cheese were collected from different sellers across Sarab. They were first evaluated for contamination by coagulase positive *Staphylococcus aureus* through culturing method and then, the isolates were subjected to PCR method according to *nuc* gene in order to confirm *Staphylococcus aureus* and methicillin resistance gene (*mecA*). The cultured samples indicated that 22 (44%) of raw milk samples, 4 (8%) of pasteurized milk samples, 9 (18%) of traditional butter samples and 12 (24%) of traditional cheese samples were contaminated by coagulase positive *S. aureus*. According to PCR using *nuc* gene primer, 18 (82%) of raw milk isolates, 4 (100%) of pasteurized milk isolates, 9 (75%) of traditional cheese isolates and 7 (78%) of traditional butter isolates contained *nuc* gene. According to PCR using *MecA* gene primer, 2 (50%) of pasteurized milk isolates and 2 (22%) of traditional cheese isolates contained *MecA* gene. No *MecA* gene was detected in raw milk and traditional butter isolates. The results provided evidence that the presence of coagulase positive *Staphylococcus aureus* and MRSA have become remarkably widespread in dairy product samples. This calls for better control of the sources of food contamination as well as the spread of antimicrobial-resistance organisms.

**Key words:** Methicillin-resistant *Staphylococcus aureus*, raw milk, dairy products, PCR, Iran

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### INTRODUCTION

Staphylococci are a group of resistant bacteria with high dispersion. They are among the first known human pathogens that can be colonized on skin and mucous membranes (Japooni *et al.*, 2004). Among the different species of this kind, *Staphylococcus aureus* is the most important type of pathogens that due to its innate ability and its capacity to acquire resistance against the antibiotics has turned into one of the major concerns about public health (Rahimi *et al.*, 2008).

*Staphylococcus aureus* is one of the most common and economically important factors in intramammary infections in dairy herds (Cabral *et al.*, 2004; Katsuda *et al.*, 2005) and has been reported to be the factor behind 30-40% of mastitis (Devriese *et al.*, 1972). On the other hand, this microorganism is the natural flora in 30-80% of the population (Scherrer *et al.*, 2004). Therefore, this microorganism can contaminate the milk

by udders with clinical or subclinical staphylococcal mastitis or from the environment during manipulation and processing. It can easily grow and reproduce inside milk and related products and may cause pathogenic enterotoxin (Rahimi *et al.*, 2008).

Food borne diseases are the main factors in the health and hygiene of the society and *Staphylococcus aureus* ranks the third as the factor beyond food borne diseases (Boerema *et al.*, 2006). Staphylococcal poisoning results from the consumption of food containing 20 to <1000 ng of staphylocoel toxin. Depending on individual sensitivity and the amount of toxin, the clinical symptoms of the illness appears in 1-6 h time after the use of contaminated food. Milk and the related dairy products, especially the traditionally and manually produced ones are of special importance in Staphylococcal poisoning (De-Neeling *et al.*, 2007).

The increasing drug resistance of bacteria and consequently the growing resultant infections in

hospitals and the society has drawn the attention of scientific cycles. The infections from methicillin resistant staphylococcus have turned into one of the major problems in antibiotic treatment (Pereira *et al.*, 2009). Methicillin-Resistant *Staphylococcus aureus* (MRSA) was first reported in the United Kingdom (UK) in 1961 and by the mid 1990s had become a major problem world wide (Mahony *et al.*, 2005).

Isolation of MRSA from animals was first reported in 1972 following its detection in milk from mastitic cows (Devriese *et al.*, 1972). Since, then especially during the last two decades the spread of this MRSA has been increasingly reported from all over the world. It is resistant to many antibiotics including  $\beta$ -lactams, semi-synthetic penicillin, cephalosporin, carbapenems and penems (Devriese *et al.*, 1972).

MRSA has a gene that is methicillin resistant (*mecA*). It encodes a protein named PBP2a (Penicillin-Binding Protein 2a) that in joining to methicillin has a less combination capacity less than the other joined proteins (Chambers, 1997).

In sensitive bacteria, (without *mec-A*), methicillin joins PBP protein of the cell membrane with greater combination capacity. This causes a soft cell membrane and finally the death of the cell. The MSRA with the mentioned genes are resistant to many other types of antibiotics. This makes the treatment of micro organism related diseases too hard and causes a greater spread of it in the society (Pereira *et al.*, 2009). The aim of this study is to determine the prevalence rate of methicillin-resistant *Staphylococcus aureus* in raw milk, pasteurized milk, ice cream and traditional butter in Sarab by culture and PCR techniques.

## MATERIALS AND METHODS

**Sampling:** In the period from March 2010 to September 2011, a total of 200 samples consisting of raw milk (50 samples), traditional white pickled cheese (50 samples), pasteurized milk (50 samples), ice cream (50 samples) were collected randomly from different supermarkets and retailer shops in Sarab. The samples were transported in 250-300 g quantities to the laboratory under refrigerated ( $5\pm 1^\circ\text{C}$ ) conditions and analyzed upon arrival.

**Bacterial isolation and identification:** For this purpose, 25 mL  $\text{g}^{-1}$  of raw milk, pasteurised milk, traditional white pickled cheese or ice cream samples were homogenized for 2 min with 225 mL of physiological sterile saline in a stomacher, Lab-Blender (PBI International, Milan, Italy).

Then, 1 mL of this suspension was added to 10 mL of cooked meat and was incubated for 24 h at a temperature of  $37^\circ\text{C}$ .

Then, 0.5 mL of the produced culture was spread over Baird-Parker agar supplemented with egg yolk and potassium tellurite and was incubated for 35-48 h at a temperature of  $37^\circ\text{C}$  (Tamagnini *et al.*, 2006). Typical coagulase-positive *S. aureus* colonies are black, grey or white and are surrounded by an opaque halo of precipitation which signifies the coagulase reaction (O'Brien *et al.*, 2009). Characteristic colonies were identified by conventional methods including gram stain, catalase test, anaerobic utilization of glucose and manitol and coagulase test (Iurlina and Fritz, 2004).

**DNA isolation for PCR:** DNA was extracted according to Cremonesia *et al.* (2005) from 1 mL of over night grown bacterial culture incubated in BHI broth at  $37^\circ\text{C}$ .

**PCR for *nuc* gene detection:** The *nuc* gene was amplified with the following two oligonucleotides: forward primer (5'-GCG ATT GAT GGT GAT ACG GTT-3') and backward primer (5'-CCAAGCCTTGACGA ACTAAAGC-3') which gave a PCR product of 255 bp.

The PCR was performed with an initial denaturation step of 3 min  $94^\circ\text{C}$  followed by 40 cycles of 60 sec  $94^\circ\text{C}$ , 60 sec  $55^\circ\text{C}$ , 60 sec  $72^\circ\text{C}$  and the extension step of 10 min  $72^\circ\text{C}$ . Agarose gels were prepared with TAE buffer (Tris, glacial acetic acid, EDTA, pH 8) and added ethidium bromide (1  $\mu\text{g}/15$  mL gel).

PCR product (5  $\mu\text{L}$ ) of each sample was mixed with 1  $\mu\text{L}$  of sample buffer (6X: 0.25% bromophenol, 0.25% xylene cyanol, 15% ficol 400) and loaded on 1.5% agarose and electrophoresed in 80 V for 60 min. The band of fragment was observed by UV transilluminator and was later documented by gel analyser machine (Kim *et al.*, 2001).

**PCR for *mec-A* gene detection:** The *mecA* gene was amplified with the following two oligonucleotides: forward primer 1276 (5'-AAA ATC GAT GGT AAA GGT TGG C-3') and backward primer 1787 (5'-AGT TCT GGA GTA CCG GAT TTG C-3') which gave a PCR product of 533 bp (Merlino *et al.*, 2002). The PCR was performed with an initial denaturation step of 3 min  $94^\circ\text{C}$  followed by 40 cycles of 60 sec  $94^\circ\text{C}$ , 30 sec  $50^\circ\text{C}$ , 90 sec  $72^\circ\text{C}$  and the extension step of 10 min  $72^\circ\text{C}$ .

Agarose gels were prepared with TAE buffer (Tris, glacial acetic acid, EDTA, pH 8) and added ethidium bromide (1  $\mu\text{g}/15$  mL gel). PCR product (5  $\mu\text{L}$ ) of each

sample was mixed with 1 µL of sample buffer (6X: 0.25% bromophenol, 0.25% xylen cyanol, 15% ficol 400) and loaded on 1.5% agarose and electrophoresed in 75 V for 60 min. The band of fragment was observed by UV transilluminator and was documented by gel analyser machine (Zamani *et al.*, 2007).

**RESULTS AND DISCUSSION**

Using culturation method, 22 (44%) of raw milk samples, 4 (8%) of pasteurized milk samples, 9 (18%) of traditional butter samples and 12 (24%) of traditional cheese were contaminated with coagulase-positive *S. aureus*. According to Polymerase Chain Reaction using *nuc* gene primer, 18 (82%) of raw milk isolates, 4 (100%) of pasteurized milk isolates, 9 (75%) of traditional cheese and 7 (78%) of traditional butter samples contained *nuc* gene (Table 1 and Fig. 1). According to Polymerase Chain Reaction using *MecA* gene primer, 2 (50%) of pasteurized milk isolates and 2 (22%) of traditional cheese contained *MecA* gene.

No *MecA* gene was detected in raw milk and traditional butter isolates (Table 1 and Fig. 2). The researchers found that according to culturation method, 22 (44%) of raw milk samples, 4 (8%) of pasteurized milk samples, 9 (18%) of traditional butter samples and 12 (24%) of traditional cheese were contaminated with coagulase-positive *S. aureus*. According to Polymerase Chain Reaction using *nuc* gene primer, 18 (82%) of raw milk isolates, 4 (100%) of pasteurized milk isolates, 9 (75%) of traditional cheese and 7 (78%) of traditional butter samples contained *nuc* gene (Table 1 and Fig. 1).

Aragon-Alegro *et al.* (2007) upon the analysis of 172 food samples including milk, soft cheese, hard cheese, ice-cream, yoghurt and fast food like sandwiches in Botucitu market in Brazil reported that 26 samples (15.1%)

of the tested food were contaminated to coagulase-positive *staphylococcus aureus*. Saei *et al.* (2009) tested 370 milk samples from cows with clinical breast inflammation in five cow breeding centers in West Azerbaijan and East Azerbaijan using culture, biochemical tests and polymerase chain reaction procedures and out of the total number of samples could identify 58 isolated *staphylococcus auras*. Akineden *et al.* (2008) collected and tested 181 samples of goat milk from Hesse market in Germany. They reported that 14 samples (17.7%) were contaminated with coagulase-positive *staphylococcus aureus* (>10 Cfu g<sup>-1</sup>) (Akineden *et al.*, 2008).

Table 1: The frequency of coagulase-positive *S. aureus* in the samples based on culture method and *nuc* and *mecA* genes in isolates

| Samples            | Number | A  | B  | C |
|--------------------|--------|----|----|---|
| Raw milk           | 50     | 22 | 18 | 0 |
| Pasteurise milk    | 50     | 4  | 4  | 2 |
| Traditional cheese | 50     | 12 | 9  | 2 |
| Traditional butter | 50     | 9  | 7  | 0 |
| Total              | 200    | 47 | 38 | 4 |

A: The number of samples contaminated to coagulase-positive *S. aureus* based on culture method, B: The number of samples containing *nuc* gene, C: The number of samples containing *MecA* gene

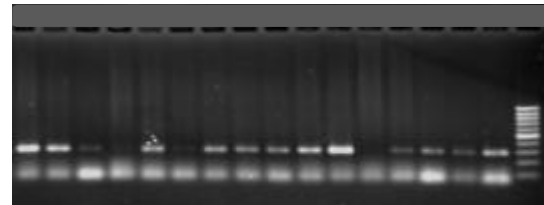


Fig. 1: Agarose gel electrophoresis analysis for the *nuc* gene in *Staphylococcus aureus* isolates. Lane 17, molecular size markers; lane 16, *S. aureus* PTCC 1112 (positive control); lanes 1-15 *S. aureus* isolates. Arrow indicates the 255 bp amplicon

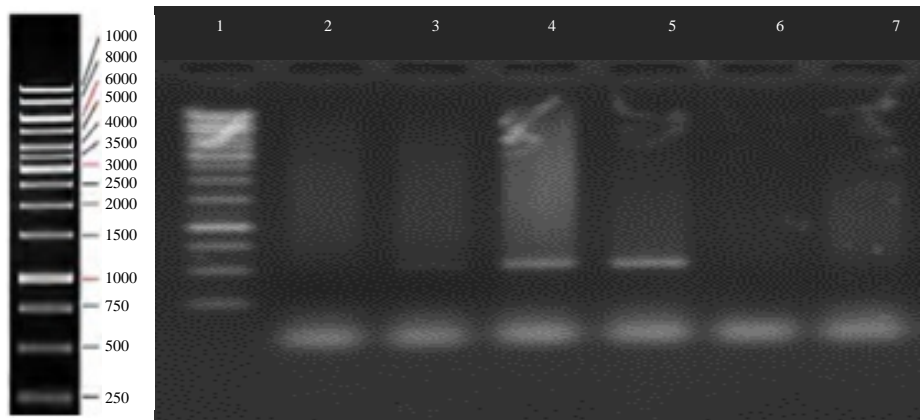


Fig. 2: Agarose gel electrophoresis analysis for the *mec-A* gene in *Staphylococcus aureus* isolates. Lane 1, molecular size markers; lanes 2, 3, 4, 5, 6 and 7 *S. aureus* isolates. Arrow indicates the 533 bp amplicon. Presence of the 533 bp shows the *mec-A* gene existence in the isolates

Normanno *et al.* (2007) tested 1634 samples including 641 dairy products and 993 meat products. They reported that 109 samples (17%) of dairy products and 100 samples (10%) from meat products and 209 (12.8%) out of the total samples were contaminated with *staphylococcus aureus*. Pereira *et al.* (2009) did polymerase chain reaction tests coagulase based on nuc and 16SrRNA on 147 coagulase-positive *staphylococcus aureus* confirmed using microbial culture and biochemical tests and they reported 100% of agreement rate between PCR and culture method.

The results of the present study indicated that according to Polymerase Chain Reaction using *MecA* gene primer, 2 (50%) of pasteurized milk isolates and 2 (22%) of traditional cheese contained *MecA* gene. No *MecA* gene was detected in raw milk and traditional butter isolates (Table 1 and Fig. 2). There have been divergent reports of the frequency of *mecA* gene among *Staphylococcus aureus* strains in Iran and the other parts of the world. This difference can be attributed to differential distribution of the mentioned gene in different places or to the method of determining them. Normanno *et al.* (2007) reported that out of the total sample of 1634 dairy and meat products, 6 samples (3.75%) were reported to have methicillin resistant *staphylococcus aureus*. Mahony *et al.* (2005) used PCR method based on *mec-A* gen and disc agar-diffusion method to study the extent of the prevalence rate of MRSA among animals and the veterinary workers in Ireland. According to their report, 25 animals including 14 dogs, 8 horses, 1 cat, 1 rabbit and 1 pig as well as 10 veterinary workers were infected with MRSA. De-Neeling *et al.* (2007) studied on 540 pigs in 9 slaughter houses in Holland and reported a high prevalence rate (39%) of infection to MRSA among the pigs.

In another research, Zamani *et al.* (2007) showed that out of a total of 70 *Staphylococcus aureus* isolates obtained from the patients who consulted with the clinical centers of Hamedan Medical Science University and a private laboratory in Iran, 50% of the strains (35 cases) in PCR method and 31.4% (22 cases) in antibiotic resistance patterns with disc agar diffusion method were resistant to methicillin. Nafisi *et al.* (2008) researched on 52 isolates of coagulase positive *Staphylococcus aureus* among 204 clinical staff workers of the different departments of Hajar Educational Institute, a subpart of Shahre Kord Medical Science University using agar screen and duplex PCR methods and reported that phenotypically 23 cases (44%) of the isolates and genotypically (*mec-A*) 27 cases of the isolates (52%) are resistant to methicillin. As MRSA may be present in raw milk and traditional dairy products, insufficiently hygienic handling of these contaminated foods may lead to transmission of MRSA to humans and possible colonization of nostrils, skin and gastrointestinal tract. Two outbreaks of MRSA after consumption of contaminated foods have been described. In the first

outbreak food contaminated by a health-care worker in a hospital most likely caused a case of MRSA septicaemia. The outbreak strain was further transmitted by a colonized nurse, causing an explosive outbreak (Kluytmans *et al.*, 1995). The second outbreak described was a typical *S. aureus* food poisoning caused by enterotoxin C producing MRSA. Isolates from stools of patients, samples of coleslaw and from a nasal swab of a food preparer were indistinguishable by PFGE (Jones *et al.*, 2002; De Boer *et al.*, 2009).

## CONCLUSION

Measures to prevent contamination and growth of MRSA in foods, including the use of microbiological criteria should be equal to those that are valid for *S. aureus* in general. Food handlers should take appropriate measures to prevent the spread of MRSA by contaminated raw foods and to prevent the occurrence, growth and survival of MRSA in prepared food. Better knowledge on transmission routes of MRSA in the food chain to provide the tools for preventing the spread of MRSA is highly needed and a proper risk assessment should be conducted to further clarify the possible health hazard for consumers related to the presence of MRSA in foods.

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