# Isolation, antimicrobial susceptibility and *mecA* gene analysis of methicillin-resistant *Staphylococcus aureus* in Iranian white cheeses

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## Summary

The objective of this study was to determine the occurrence of antimicrobial resistance among *Staphylococcus aureus* and to estimate the presence of methicillin-resistance in *S. aureus* (MRSA) isolates obtained by culture and polymerase chain reaction (PCR) methods. For this purposes, 100 Iranian white and feta cheese samples collected from different suppliers were initially evaluated for the occurrence of *S. aureus* by culturing methods. The obtained isolates were subjected to disc diffusion antimicrobial susceptibility tests and a PCR method to detect the *mecA* gene. Out of the 100 cheese samples examined, 25 (25%) samples were contaminated with *S. aureus* with a mean of 5.74 ± 5.67 log cfu/g. Out of the 25 isolates, 23 (92%) were found to be resistant to at least one antibiotic or more, tested by a disk diffusion method. The highest rate of antibiotic resistance was observed to penicillin G (92%) followed by ampicillin (73%) and cloxacillin (68%). None of the isolates was resistant to gentamycin and vancomycin. Eight (34.78%) of the 23 *S. aureus* isolates were genotypically confirmed as MRSA. The results indicate that the presence of antimicrobial resistant strains of *S. aureus* in Iranian cheese samples constitute a potential risk for human health. This calls for better control of the spread of antimicrobial resistant strains as well as cheese contamination sources.

**Key words:** Methicillin-resistant *Staphylococcus aureus* (MRSA), Iranian white cheese, PCR, Iran

## Introduction

*Staphylococcus aureus* is an opportunistic human pathogen considered as the third most common pathogen causing food poisoning in the world. Staphylococcal food poisoning, which is caused by the ingestion of food that contains enterotoxins (Scherrer *et al*., 2004), is one of the most prevalent causes of gastroenteritis worldwide. *Staphylococcus aureus* can gain access to milk either by direct excretion from udders with clinical or subclinical mastitis or by cross contamination and raw milk processing (Scherrer *et al*., 2004; Jorgensen *et al*., 2005). Its presence in raw milk is a major concern for the safety and quality of traditionally produced cheeses (Delbes *et al*., 2006).

Cheese, particularly Iranian Feta cheese, is an integral part of the Iranian diet, which has an annual consumption per capita of 5.4 kg (Alizadeh *et al*., 2005). Iranian white cheese is a local brined cheese that is produced traditionally throughout Iran. It is a close textured brined cheese made from unpasteurized cow's milk, sheep's milk or mixtures of both without the addition of a starter culture. Its characteristic flavor, body and texture are developed during the ripening period from several weeks to months. Iranian Feta cheese made from bovine milk is manufactured in modern dairy plants from ultrafiltered and pasteurized milk with mesophilic starter cultures and commercial microbial rennet. The main characteristics of this type of cheese include a minimum of 34% (w/w) total solids, a fat content of 15%, a protein content of 11% and a pH of 6.20-6.65.

*Staphylococcus aureus* is able to produce a wide range of extracellular toxins and virulence factors that contribute to causes of disease (Haveri *et al*., 2007). Among the virulence factors of *S. aureus*, antibiotic resistance plays an important role. Numerous bacteria are resistant to antibiotics because of the extended use and misuse of antibiotics in the treatment of animal and human diseases. In the last decade, antibiotic resistance in bacteria has caused increased concerns about public health. *Staphylococcus aureus* strains can be characterized by single or multiple antibiotic resistance and represent a major threat to public health (Pereira *et al*., 2009). Methicillin-resistance in staphylococci is mediated by the *mecA* gene, encoding the penicillin binding protein 2a (PBP2a), which has a reduced affinity for β-lactams (Normanno *et al*., 2005; Haenni *et al*., 2010). Therefore, the *mecA* gene is considered as a useful molecular marker of methicillin-resistance in all staphylococci. Other chromosomally determined factors, such as the *femA* operon that act as regulator genes, are essential for the expression of methicillin-resistance in *S. aureus* (Vannuffel *et al*., 1995). In fact, the simultaneous detection of the *femA* and *mecA* genes is advantageous in identifying both species and genotypic resistance of

staphylococci.

The main objectives of the present study were to enumerate *S. aureus* in Iranian white cheeses and to detect the *femA* and *mecA* genes in methicillin-resistant isolates from cheese.

## Materials and Methods

One hundred cheese samples (50 traditional white cheese and 50 Iranian Feta cheese samples produced by traditional and industrial methods) were collected from various dairy supplying centers in Mashhad, Iran. All samples were placed in sterile plastic bags and kept at +4°C prior to the analysis, which began immediately after transporting the samples to the laboratory, usually at the same day.

### Isolation and identification of *S. aureus*

The samples (10 g) were weighed, put into sterile stomacher bags, diluted with 90 ml of 2% sterile sodium citrate, and homogenized in a BagMixer® (Interscience) for 1-2 min.

Isolation of *S. aureus* from cheese samples was performed according to ISO 6888-1 (Anonymous, 1999) using Baird Parker Agar (Oxoid) supplemented with egg yolk tellurite emulsion (Oxoid), 0.1 ml of the dilution levels' portion were streaked on the Baird-Parker agar (Oxoid) and incubated at 37°C for 30 to 48 h. From each plate, typical colonies with similar morphologies, were taken for confirmation testing and cultured on Brain Heart Infusion agar (Oxoid). *S. aureus* isolates were identified by conventional methods, including Gram staining, production of coagulase, catalase, DNAse (DNase agar, Oxoid), and aerobic production of acid from mannitol (Mannitol salt agar, Oxoid) and other biochemical tests. The strains identified as *S. aureus* were kept frozen at -20°C in Trypticase soy Broth (Oxoid) containing 15% (v/v) glycerol until molecular tests were carried out.

### Antimicrobial susceptibility testing

Antibiotic resistance of isolated *S. aureus* was tested by applying a disk diffusion assay according to the guidelines of NCCLS (NCCLS, 2003) using Muller Hinton agar (Oxoid). All the identified *S. aureus* were tested for penicillin G (10 IU), ampicillin (10 µg), amoxycillin (25 µg), oxacillin (1 µg), streptomycin (10 µg), methicillin (5 µg), tetracycline (30 µg), cephalotin (30 µg), cloxacillin (5 µg), gentamycin (10 µg), vancomycin (30 µg), erythromycin (15 µg), chloramphenicol (30 µg) and cotrimoxazole (1.25/23.75 µg). A methicillin susceptible *S. aureus* strain (ATCC 25923) and a methicillin-resistant *S. aureus* (ATCC 43300) were used as negative and positive controls, respectively. Zones of growth inhibition were measured after overnight incubation and the resistance or susceptibility of the antibiotics was interpreted as suggested by standards (NCCLS, 2003).

### Detection of the *mecA* and *femA* by PCR

Total genomic DNA was extracted from an overnight culture of each isolate in Brain Heart Infusion Broth (BHI, Oxoid) at 35°C using Genomic DNA purification kit (Bioneer, Korea). On the basis of the DNA sequences of the *femA* gene, the previously described primers,were used: primer femA-F (5′-GCA AAC TGT TGG CCA CTA TG-3′) and femA-R (5′-TCA TCA CGA TCA GCA AAA GC-3′) which amplified a 594 bp fragment of the *femA* gene (Riyaz-Ul-Hassan *et al*., 2008). The presence of *mecA* gene (533 bp) was detected by PCR as described by Lee (2006). The DNA of the putative MRSA strains was amplified with the primers mecA-F (5′-AAA ATC GAT GGT AAA GGT TGG C-3′) and mecA-R (5′-AGT TCT GCA GTA CCG GAT TTG C-3′). PCR was performed in a 25 µl volume. The reaction mix contained 10 mM Tris-HCl (pH = 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, a 200 µM concentration of each dNTP, 2.5 U of *Taq* DNA polymerase and a 0.2 µM concentration of each primer. The PCR thermal program was modified and amplification was performed under the following conditions: initial denaturation at 94°C for 5 min followed by denaturation at 94°C for 60 s, annealing at 55°C for 30 s and primer extension at 72°C for 60 s, with a total of 35 cycles and an additional extension at 72°C for 10 min. Amplified DNA fragments were visualized under UV transillumination following electrophoresis on 1.5% agarose gel stained with ethidium bromide. The Gene Ruller Plus 100 bp DNA Ladder (Cinagen Ltd, Iran) was used as a reference standard.

## Results

Twenty five (25%) out of 100 samples were contaminated with *S. aureus* with a mean of 5.74 ± 5.67 log cfu/g. Of the 50 Iranian Feta cheese samples and the 50 traditional white cheese samples examined, 7 (14%) and 18 (36%) were contaminated with *S. aureus*, respectively. The cheese samples tested were contaminated with *S. aureus* from 3 log to 6.47 log cfu/g (Table 1).

Regarding antimicrobial susceptibility testing, 23 (92%) out of 25 *S. aureus* isolates from 100 cheese samples were found to be resistant to at least one or

**Table 1:** Occurrence of *S. aureus* isolates in cheese samples

| Cheese samples | No. of samples | Positive samples (%) | Mean±SE of contamination (log cfu/g) | No. (%) of *mecA* positive |
|---|---|---|---|---|
| Iranian Feta cheese | 50 | 7 (14) | 4.82 ± 3.80 | 2 |
| Traditional white cheese | 50 | 18 (36) | 6.01 ± 4.36 | 6 |
| Total | 100 | 25 (25) | 5.74 ± 5.67 | 8 |

**Table 2:** Antimicrobial sensitivity of *S. aureus* isolates (n=25)

| Antibiotics | Disc content | Resistant No. (%) | Intermediate No. (%) | Sensitive No. (%) |
|---|---|---|---|---|
| Penicillin G | 10 Units | 23 (92) | 0 | 2 (8) |
| Ampicillin | 10 µg | 18 (73) | 0 | 7 (28) |
| Amoxycillin | 25 µg | 5 (20) | 2 (8) | 18 (72) |
| Methicillin | 5 µg | 8 (32) | 0 | 17 (68) |
| Cephalotin | 30 µg | 9 (36) | 0 | 16 (64) |
| Oxacillin | 1 µg | 8 (32) | 0 | 17 (68) |
| Tetracycline | 30 µg | 11 (44) | 3 (12) | 11 (44) |
| Cloxacillin | 5 µg | 17 (68) | 0 | 8 (32) |
| Streptomycin | 10 µg | 6 (24) | 0 | 19 (76) |
| Cotrimoxazole | 1.25/23.75 µg | 2 (8) | 0 | 23 (92) |
| Erythromycin | 15 µg | 3 (12) | 5 (20) | 17 (68) |
| Chloramphenicol | 30 µg | 6 (24) | 0 | 19 (76) |
| Gentamycin | 10 µg | 0 | 0 | 25 (100) |
| Vancomycin | 30 µg | 0 | 0 | 25 (100) |

more antibiotics tested by disk diffusion method (Table 2).

As shown in Table 2, the highest antibiotic resistance rate was to penicillin G (92%) followed by ampicillin (73%), cloxacillin (68%), tetracycline (44%), cephalotin (36%), methicillin and oxacillin (32%), streptomycin and chloramphenicol (24%), erythromycin (12%) while the lowest resistance rate was to cotrimoxazole (8%). None of the strains was resistant to gentamycin and vancomycin.

In this study, out of 23 resistant isolates, 1 isolate (4.35%) to two antibiotics, 5 (21.74%) to three antibiotics, 5 (21.74%) to four antibiotics, 2 (8.7%) to five antibiotics and 10 (43.48%) to six and more antibiotics were resistant simultaneously (Table 3).

**Table 3:** Frequency and percentage of multi-resistance patterns of *S. aureus* isolates (n=23) for selected antimicrobial agents

| Antibiotic | Frequency | Resistant (%) |
|---|---|---|
| One antibiotic | 0 | 0 |
| Two antibiotics | 1 | 4.35 |
| Three antibiotics | 5 | 21.74 |
| Four antibiotics | 5 | 21.74 |
| Five antibiotics | 2 | 8.7 |
| Six and above | 10 | 43.48 |

Eight (34.78%) out of 23 resistant isolates were identified as MRSA *S. aureus* by disk diffusion method. These isolates were also found to be *mecA* positive and genotypically confirmed as MRSA (Fig. 1). In this study, two MRSA strains from white cheese and 6 MRSA strains from fresh soft cheese were isolated. As an internal control, the 594 bp product of *femA* was detected in all isolates, confirming the presence of *S. aureus* and validating the PCR protocol.

## Discussion

Similar to *Clostridium perfringens*, *Staphylococcus aureus* enterotoxins appear as the second most frequent cause of milk-borne disease outbreaks in several developing countries, after *Salmonella* (De Buyser *et al*., 2001).

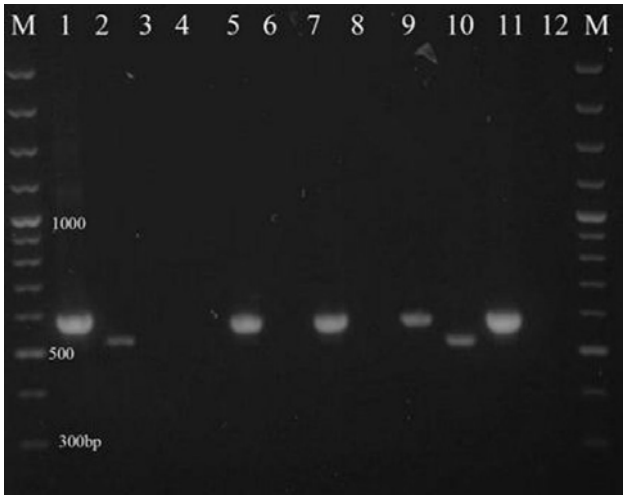


**Fig. 1:** Typical amplicons of *femA* and *mecA* genes of some isolates. M: 100 bp standard marker. Line 1: *femA* and Line 2: *mecA* of *S. aureus* ATCC 43300, Lines 3 and 4: Negative control, Line 5: *femA* positive and Line 6: *mecA* negative of *S. aureus* ATCC 25923, Lines 7 and 11: *femA* positive of Methicillin-sensitive *S. aureus* isolate, Line 9: *femA* and Line 10: *mecA* positive of Methicillin-resistant *S. aureus* isolate, and Line 12: *mecA* negative of *S. aureus* isolate

Different from previously performed research, in our study, 25 (25%) out of the 100 samples of Iranian Feta and traditional white cheeses were contaminated with *S. aureus*. Marhamatizadeh *et al*. (2006) reported an occurrence of 46% in traditional cheese samples while Fooladi *et al*. (2010) found that 10% of their samples was contaminated with *S. aureus*.

Another study revealed an *S. aureus* prevalence of 26% and 16% isolated from the tested cheese and butter samples (Mirzaei *et al*., 2012). Our finding is in agreement with the results of the latter study.

Jorgensen *et al*. (2005) reported an occurrence of 75%, while Gundogan *et al*. (2005) demonstrated a prevalence of 94.5% in *S. aureus* isolated from milk and ice cream. Can and Celik (2012) reported that out of 200 Turkish opening cheese samples examined, 9.5% were contaminated with *S. aureus*. Akineden *et al*. (2008) noted that 7.7% out of a total of 181 cheese samples prepared in various European countries were con-

taminated with *S. aureus*.

These different results may be based on the differences in cheese production technologies, the number of samples and whether the used milk was raw or pasteurized. It could also be related to the level of hygiene where the cheese is produced and the personnel involved in production.

*Staphylococcus aureus* has developed β-lactam resistance worldwide, although reported prevalence rates indicate that wide variations exist regionally (De Oliveira *et al*., 2000; Normanno *et al*., 2007). Depending on the origin of the sample, the prevalence of β-lactam resistant strains of *S. aureus* are extremely different (Stephan *et al*., 2001; Jorgensen *et al*., 2005; Anderson *et al*., 2006).

In this study, all isolates (100%) were resistant to one or more of the examined antibiotics. The highest antibiotic resistance rate was observed to penicillin G (92%) followed by ampicillin (73%) and the lowest rate of resistance to cotrimoxazole (8%). This finding is in agreement with Bartolomeoli *et al*. (2009) and Ebrahimi and Akhavan Taheri (2009), who demonstrated that *S. aureus* isolates were resistant to cloxacillin (100%), penicillin (87%), and ampicillin (62.5%). Our findings are also in agreement with those reported from cows with mastitis in Argentina (40%) and Tehran (57%) by Gentilini *et al*. (2002) and Gooraninejad *et al*. (2007), respectively.

In the present study, all investigated isolates were sensitive to gentamicin and vancomycin. This finding is consistent with other works (Normanno *et al*., 2007; Bartolomeoli *et al*., 2009; Can and Celik, 2012). Andre *et al*. (2008) also found all *S. aureus* strains isolated from cheese samples to be sensitive to vancomycin and gentamycin. Kaszanyitzky *et al*. (2004) reported that 96, 55 and 45% of the *S. aureus* isolates recovered from humans, bovine mastitis and foods tested positive for β-lactamase. These data call for a policy regarding the accurate use of antibiotic components in Iranian dairy production.

This study also tried to show the multi-drug resistance profile of *S. aureus* isolated from cheese samples. From 23 resistant isolates, 4.35, 21.74, 21.74 and 8.7% of the isolates were resistant to two, three, four and five antibiotics, respectively, and 43.48% of isolates to six and more antibiotics (Table 3). The multi-drug resistance profile found in this study is in agreement with that of Sharma *et al*. (2011) who found that *S. aureus* was resistant to several antibiotics. A similar result was found by Shitandi and Sternesjo (2004) in milk from large and small-scale producers in Kenya. They reported that 34.3% of the isolates from small scale and 18% of isolates from large scale producers showed multiple drug resistance (≥2 antibiotics). Differences in the resistance rate of various antibiotics could result from the difference in using various antibiotics in various countries and the isolated strains. Inappropriate use of antibiotics is suspected to be a major contributing factor to the relatively high level of resistance to β-lacatms observed in this study.

Methicillin-resistant *S. aurues* infection is a global health issue due to the severity of illnesses it may cause.

In the present study, methicillin-resistant *S. aureus* was detected in an average of 32% in 100 samples. Normanno *et al*. (2007) isolated 160 *S. aureus* from food samples with animal origins in Italy and found 6 strains to be *mecA* positive. In the same way, MRSA strains were found to be enterotoxigenic and showed resistance to at least one of the antibiotics tested. In a study in Turkey, Can and Celik (2012) isolated 12 *S. aureus* strains from Turkish cheese samples and found two to be *mecA* positive.

In the present study, *femA* and *mecA* genes were targeted for the detection of *S. aureus* by PCR. The inclusion of an internal positive control (*femA*) in the reaction provides assurance against false-negative results. As an internal control, *femA* was found to be present in all of the isolates studied.

In conclusion, because *S. aureus* is one of the most important pathogens responsible for food intoxication, the presence of multiresistant strains in the community, particularly in countries where antibiotic availability and use is not well regulated, is a major public health problem. It was found that the majority of the isolates were susceptible to various antibiotics especially gentamycin and vancomycin. Penicillin G, ampicillin, cloxacillin and tetracycline were observed to be less effective against *S. aureus* in Iranian white cheese.

MRSA is a major health concern to humans and animals alike. The present study provides an overview on the MRSA situation in cheese products in Iran. However, little data was available on the occurrence and characteristics of MRSA in the country; therefore, direct comparison of results was not possible. The results were hence compared with those from other countries considered to be the most appropriate and relevant to the present study.

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