RESEARCH ARTICLE

•Arch Hyg Sci 2016;5(4): 302-309
•Journal Homepage: http://jhygiene.muq.ac.ir

Detection of Methicillin-Resistance Gene in *Staphylococcus aureus* Isolated from Traditional White Cheese in Iran

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A-R-T-I-C-L-EI-N-F-O

Article Notes:
Received: Mar 25, 2016
Received in revised form: Jul 29, 2016
Accepted: Sep 25, 2016
Available Online: Sep 28, 2016

Keywords:
Staphylococcus aureus
Methicillin-resistant gene
white cheese
PCR
Iran

A-B-S-T-R-A-C-T

Background & Aims of the Study: Methicillin-resistant *staphylococcus aureus* (MRSA) is considered as a major pathogen in public health concern. The objectives of this study were to firstly determine antibiotic sensitivity among *Staphylococcus aureus* isolated from traditional Iranian white cheese during 2015 from Hamedan province of Iran; and secondly to estimate the presence of methicillin-resistant *S. aureus*.

Materials &Methods: This cross-sectional study was done by collecting 120 Iranian white cheeses (traditional and industrial) which were available in different markets; and tested for the presence of *S. aureus* by culture methods. The obtained isolates were subjected to disc diffusion antimicrobial susceptibility tests followed by PCR detection of the *mecA* gene.

Results: Out of 120 examined cheese samples, 19 samples (31.67%) were contaminated with *S. aureus*. The highest rate of antibiotic resistance was observed for penicillin, as all of the 19 isolates (100%) were found to be resistant to this antibiotic using disk diffusion method. Three out of 19 *S. aureus* isolates (15.7%) were phenotypically resistant to methicillin (disk diffusion), while 4 (21.05%) of them were genotypically confirmed as MRSA strains. Furthermore, none of the isolates were found resistant to vancomycin.

Conclusion: The results of the study confirm the presence of methicillin resistant strains of *S. aureus* in Iranian white cheese. It should be considered to constitute a potential health risk for consumers, suggesting usage of more stringent hygiene measures.

Please cite this article as: Varmazyar-najafi M, Pajohi-alamoti M, Mohammadzadeh A, Mahmoodi P. Detection of Methicillin-Resistance Gene in *Staphylococcus aureus* Isolated from Traditional white cheese in Iran. Arch Hyg Sci 2016;5(4):302-309.

Background

Cheese, particularly Iranian white cheese, which is an integral part of the Iranian diet, has an annual consumption per capita of 5.4 kg (1). Iranian white cheese is a local brined cheese which is traditionally produced throughout the country. This close textured brined cheese is made from unpasteurized cow and sheep's milk or mixtures of both without addition of any starter culture. Its flavor, body and texture are developed during the ripening period taking several weeks or months. Industrial Iranian white cheese is also made from ultra-filtered and pasteurized cow's milk using mesophilic starter cultures and commercial microbial rennet. The main characteristics of this type of cheese are a minimum of 34% (w/w) total solids, a fat content of 15%, a protein content of 11% and a pH of 4.20-4.65. As expected, cheese may be contaminated with different types of bacteria; among which *Staphylococcus*

aureus is one of the most important. This bacterium can gain an access to milk either by direct excretion from udders with clinical or subclinical mastitis, or by cross contamination and raw milk processing (2,3).

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 ingestion of foods containing enterotoxins, is one of the most prevalent causes of gastroenteritis worldwide (2-4). Moreover, because of the expanded use and misuse of antibiotics in the treatment of human and animal diseases, antibiotic resistance is increasing as a major concern for public health. On the other hand, high consumption of antibiotic in the community can be a major problem in treatment. *Staphylococcus aureus* strains may show single or multiple antibiotic resistances representing a major threat for human and animals' health (4-8). B-lactams (e.g. penicillins, cephalosporins) are some major groups of antibiotics that because of their massive use, antibiotic resistance against them has continually been increasing. Methicillin is one of latest antibiotics of this group and resistance against it can be very important. The *Staphylococcus aureus* isolates are divided into two groups: methicillin-resistant *Staphylococcus aureus* (MRSA); and methicillin-susceptible *Staphylococcus aureus* (MSSA). Thus, the presence of such MRSA strains in cheese samples could be a major concern on safety and quality of traditionally produced cheese. Methicillin-resistance is mediated by the *mecA* gene which encodes a penicillin binding protein, PBP2a, with reduced affinity for β-lactams (9,10).

Therefore, the *mecA* gene is considered as a useful molecular marker to identify 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* (11), as well. The *femA* gene is also a molecular marker for genotypic identification of *S. aureus* species and is universally present in all *S. aureus* isolates (12). In fact, simultaneous detection of *femA* and *mecA* genes is advantageous in identifying the species and genotypic methicillin resistance of *S. aureus*.

Aims of the study:

This study was aimed to investigate antibiotic sensitivity and methicillin resistance gene in *Staphylococcus aureus* isolated from traditional Iranian white cheese, Hamedan province, Iran, during 2015.

Materials & Methods

Sampling: This cross-sectional study was conducted during 2015. A total of 120 cheese samples consisting traditional (60) and industrial (60) Iranian white cheese were purchased from central dairy markets in Hamedan province, located in the west of Iran. All samples were packed in sterile conditions and kept at 4°C prior for analysis, which began immediately after the transportation of samples to food hygiene laboratory, faculty of veterinary science, Bu-Ali Sina University.

Bacterial culture

Isolation of *S. aureus* from cheese samples was performed according to ISO 68881 (13). Initially, samples were homogenized, then 10 g from each sample was diluted with 90 ml of 0.1% sterile peptone water. Using Baird-Parker agar medium supplemented with egg yolk-tellurite emulsion, 0.1 ml of the dilution was streaked on the medium and incubated at 37°C for 24 to 48 h. The black colonies with bright halos were selected as *S. aureus* were identified by conventional methods, including Gram staining, production of coagulase, catalase, DNase, fermentation of mannitol and other biochemical tests. The identified strains were kept frozen at -20°C in Nutrient broth containing 30% glycerol until PCR confirming.

Extraction of DNA samples

DNA was extracted from each of the isolated *S. aureus* strains, using a previously described protocol (14). Briefly, about 3 ml of an overnight Nutrient broth culture of each of the bacterial isolates was transferred into a microtube and the bacterial cells were precipitated at 8000 rpm for 3 min. Afterward, 200 µl of a lysis buffer (1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl, 1 mM EDTA, pH=8.0) was added to the pellets and microtubes were incubated in a boiling water bath (100°C) for 10 min followed by being centrifuged at 10000 rpm for 2 min. The supernatants were transferred into clean microtubes and 3-5 µl of each sample was used as template DNA in PCR assays.

Multiplex PCR

Extracted DNA samples from *S. aureus* isolates were examined by a multiplex PCR assay targeting two genes, *femA* and *mecA*, to genetically detect *S. aureus* species and MRSA strains simultaneously. The oligonucleotide primers were previously described by Mehrotra et al. (2000) and their sequences presented in table 1. The PCR reaction (25 µl) contained 4-5 µl of template DNA, 2.5 µl of 10× PCR buffer, 0.75 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.25 µl of 5 U/µl of Taq DNA polymerase, and 10 pmol of each of the primers. The PCR amplification was performed under the following conditions: initial denaturation at 94°C for 5 min followed by denaturation at 94°C for 2 min, annealing at 57°C for 2 min, extension at 72°C for 1 min (35 cycles) and a final extension at 72°C for 7 min (12). The products of PCR were analyzed by electrophoresis on 2.5% agarose gel containing ethidium bromide (0.5 µg/ml).

Table 1) Primer pairs used in PCR assays

Primer	Sequence 5'- 3'	Product size (bp)
***mecA*-forward**	ACTGCTATCCACCCTCAAAC	163
***mecA*-reverse**	CTGGTGAAGTTGTAATCTGG	
***femA*-forward**	AAAAAAGCACATAACAAGCG	132
***femA*-reverse**	GATAAAGAAGAAACCAGCAG	

Antimicrobial susceptibility testing

Antibiotic resistance of isolated *S. aureus* strains were tested by applying a Kirby-Bauer disk diffusion assay according to the guidelines of CLSI (15). All identified *S. aureus* strains were tested for a panel of antibiotics including penicillin (10 unite), oxacillin (30 µg), streptomycin (10µg), tetracycline (30 µg), cefoxitin (30 µg), gentamycin (10 µg), vancomycin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), carbenicillin (100 µg), doxycycline (30 µg), kanamycin (30 µg), and cefixime (5µg). *S. aureus* (ATCC 33591) and a methicillin-resistant *S. aureus* (ATCC 6538) were used as negative (MSSA strain) and positive controls, respectively. Zones of growth inhibition were measured after an overnight incubation. Resistance or susceptibility to the antibiotics was interpreted as suggested by standard method.

Results

Based on the bacterial culture methods, 19 out of 120 cheese samples (15.8%) were contaminated with *S. aureus*. All of them in which they were isolated from traditional white and no *S. aureus* was seen in industrial cheese samples. Considering each group separately, out of the 60 industrial Iranian white cheese and 60 traditional white cheese samples examined, 0 (0%) and 19 (31.67%) were contaminated with *S. aureus*, respectively (Table 2).

The expected DNA fragment (132 bp), representing the presence of *femA* gene, was amplified from all of the phenotypically characterized *S. aureus* strains in PCR examination. Among these strains, the *mecA*

gene was only detected in 4 isolates by PCR and genotypically confirmed as MRSA strains (Fig. 1). However, 3 out of these 4 strains were shown to be resistant to oxacillin (a representative antibiotic for determination of resistance to methicillin) in antimicrobial sensitivity test. As shown in Table 3, the highest antibiotic resistance rate was observed against penicillin (100%), while the lowest resistance rate belonged to kanamycin (5.26%) and ciprofloxacin (5.26%). None of the strains was resistant to vancomycin. As mentioned, 3 (15.79%) out of these 19 *S. aureus* isolates were identified as MRSA strains where the bacteria showed resistance against oxacillin.

Table 2) Distribution of isolated *S. aureus* in the two sample groups

samples	No	Isolates (%)	*mecA* positive (%)
Industrial white cheese	60	0	0
Traditional white cheese	60	19 (31.67)	4 (21.05)

Table 3) Antibiotic sensitivity patterns in *S. aureus* isolated from cheese samples.

Antibiotic	Resistant N (%)	Intermediate N (%)	Sensitive N (%)
penicillin (10 U)	19 (100)	0	0
ciprofloxacin (5 µg)	1 (5.26)	2 (10.52	16 (84.21)
oxacillin (30 µg)	4 (21.05)	0	15 (78.94)
chloramphenicol (30 µg)	10 (52.36)	1 (5.26)	8 (42.10)
carbenicillin (100 µg)	3 (15.79)	5 (26.31)	11 (57.89)
gentamycin (10 µg)	4 (21.05	0	15 (78.94)
vancomycin (30 µg)	0	0	19 (100)
doxycycline (30 µg)	2 (10.52	1 (5.26)	16 (84.21)
streptomycin (10 µg)	2 (10.52)	8 (42.1)	9 (47.36)
tetracycline (30 µg)	2 (10.52)	0	17 (89.47)
cefixime (5 µg)	10 (52.36)	6 (31.57)	3 (15.78)
kanamycin (30 µg)	1 (5.26)	2 (10.52)	16 (84.21)
cefoxitin (30 µg)	2 (10.52)	2 (10.52)	15 (78.94)

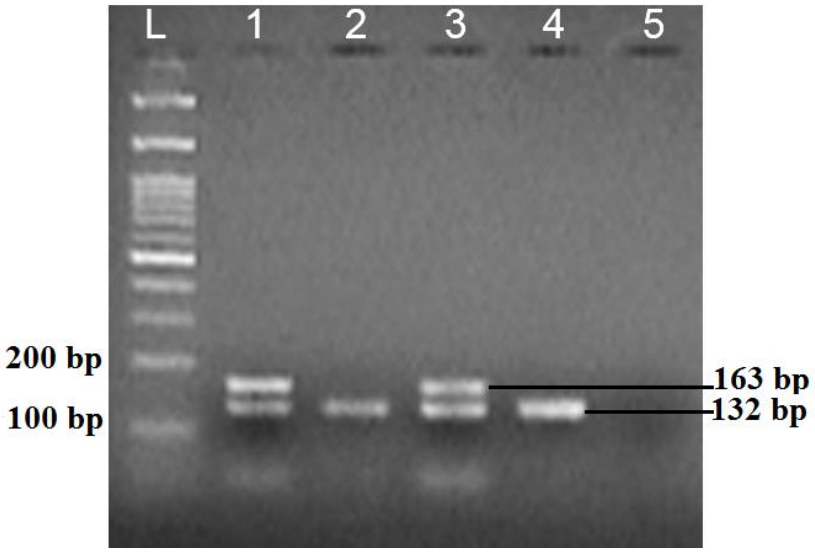

Figure 1) the results of electrophoresis of PCR products. Lane L: 100 bp DNA ladder, Lane 1: *S. aureus* (ATCC 33591) containing amplified DNA fragments from both *femA* and *mecA* genes (132 and 163 bp, respectively) as positive control. Lane 2: *S. aureus* (ATCC 6538) containing amplified DNA fragments from only *femA* gene (132 bp). Lane 3: a tested isolate which was positive for both genes. Lane 4: a *S. aureus* isolate which was negative for *mecA* gene. Lane 5: negative control containing no template DNA.

Discussion

Considering their essential nutrients, consumption of dairy products is rapidly increasing among consumers. One of these products is cheese which can provide valuable nutrients such as calcium and protein along with different tastes and flavors. Iranian white cheese is a savory type of cheeses which has a high rate of consumption. Contamination of this dairy product with *Staphylococcus aureus* is a potential concern for consumers' health, as this bacterium may cause direct infection, produces enterotoxins, and/or transfer multidrug resistance. Consequently, many studies have been conducted to detect staphylococcal contamination of food products along with the investigation of MRSA strains.

Marhamatizadeh et al. (2006) performed a study in Kazerun (Iran) and isolated 23 (46%) *S. aureus* strains from 50 traditional cheese samples which showed relatively high prevalence of the bacteria (16). Aragon-Alegro et al. (2007) examined 172 food samples including soft and hard cheeses in Brazil and found that 26 samples (15.1%) were contaminated with *S. aureus* (17). Normanno et al. (2007) tested 641 samples of dairy products in a research in Italy. Their results showed that 109 samples (17%) were contaminated with *S. aureus* (18).

In a study carried out by Mirzaei et al (2012) in Tabriz (Iran), a prevalence of 26 (26%) *S. aureus* was reported from 100 examined traditional cheese samples. In further examinations, 21 (81%) out of those 26 isolates were found to be resistant to oxacillin disk, while only 19 (73%) isolates were positive for *mecA* gene (19). In another research, they also found that 50 (50%) out of 100 raw milk samples and 2 (2%) out of 100 pasteurized milk samples were contaminated with *S. aureus*. 27 (54%) and 14 (28%) out of those 50 strains which were isolated from raw milk samples were phenotypically and genotypically confirmed as MRSA, respectively. However, the isolates from pasteurized milk did not belong to MRSA. Although, 13 (28%) out of 46 isolates harbored the *mecA* gene, none of them showed oxacillin resistance by disk diffusion

test (20). Arefi et al. (2014) isolated 25 (25%) *S. aureus* strains from 100 Iranian white and feta cheese samples in Mashhad. Out of which, 8 (32%) of them were found to be oxacillin resistant; whereas, 23 (92%) isolates were genotypically confirmed as MRSA (21). The results of such studies suggest that differences in cheese production technologies, number of samples, milk source (raw/pasteurized), and hygiene measures may impact the rate of contamination (22).

In the present study, 120 samples containing 60 traditional and 60 industrial white cheese samples were examined for *S. aureus* contamination. Although, 19 (31.67%) *S. aureus* strains were isolated from 60 traditional white cheese samples, none of the industrial white cheese samples were found to be contaminated with the bacterium. They were also confirmed to be *S. aureus* by a PCR assay using *femA* species-specific gene. The inclusion of such an internal positive control (*femA*) in the reaction provided assurance against false-negative results. All of *S. aureus* isolates were examined in the antibacterial susceptibility tests and the results showed that 100% of isolates were resistant to one or more of the examined antibiotics. Although all isolates were sensitive to vancomycin, the highest antibiotic resistance rate was observed against penicillin (100%), indicating a widespread resistance to this antibiotic which may be arisen from its wide use/misuse (23). Besides, the lowest rate of resistance was observed against kanamycin and ciprofloxacin (5.26%). Of course, 3 out of 19 *S. aureus* isolates found to be among MRSA stains as they were resistant to oxacillin disk in the performed antibacterial susceptibility tests. Nevertheless, all of *S. aureus* isolates were also investigated for the presence of *mecA* gene. Using PCR, this gene was detected in 4 (21.05%) out of 19 isolates, 3 of them were previously shown to be resistant in the antibacterial susceptibility tests. This difference may be resulted from the procedure of antibacterial susceptibility test, the lack of expression of *mecA* gene, etc (24). However, the results showed relatively high rate of MRSA strains among *S. aureus* isolates.

Conclusion

S. aureus is one of the most important pathogens which is responsible for food intoxication and the presence of multi-resistant strains in the community, particularly in countries where antibiotic availability the and use of it is not well regulated. As indicated by the results, penicillin was absolutely not effective against *S. aureus* strains isolated from the examined traditional Iranian white cheese samples. This can really be a major concern of public health as such strains may easily circulate in the human populations and cause disease and/or confer their antibiotic resistance to other bacteria in the community. On the other hand, no *S. aureus* was isolated from industrial Iranian white cheese samples; which probably signifies applying appropriate food hygienic measures during production of such kind of cheese, suggesting that they are safer for consumers. Moreover, MRSA strains were isolated in the present study. As methicillin is one of the recent available choices against staphylococcal infections, this should also be considered as a major health concern for humans and animals. The present study provides an overview on the MRSA situation in Iranian white cheeses in Hamedan, Iran. However, little data are available on the occurrence and characteristics of MRSA in Iranian white cheese; therefore, direct comparison of the results was not possible. The results were hence compared with those from other countries only assumed to be the most appropriate and relevant to the present study.

Footnotes

Acknowledgments

The authors are grateful to Bu-Ali Sina University (Hamedan, Iran) for financial support.

Conflict of Interest:

The authors declared no conflict of interest.

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