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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

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 micro organism 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). Staphylococal poisoning results from the consumption of food containing 20 to <1000 ng of staphylocoal 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 Staphylococal poisoning (De-Neeling *et al*., 2007).

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

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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 β-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±1°C) conditions and analyzed upon arrival.

Bacterial isolation and identification: For this purpose, 25 mL 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°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°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°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'-CCAAGCCTTGACGAACTAAAGC-3') which gave a PCR product of 255 bp.

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

PCR product (5 μL) 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 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°C followed by 40 cycles of 60 sec 94°C, 30 sec 50°C, 90 sec 72°C and the extension step of 10 min 72°C.

Agarose gels were prepared with TAE buffer (Tris, glacial acetic acid, EDTA, pH 8) and added ethidium bromide (1 μg/15 mL gel). PCR product (5 μL) 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 Azerbijan and East Azerbijan 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^{-1}) (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

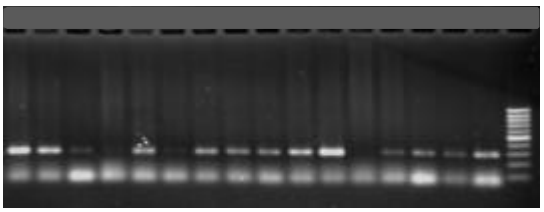

Fig. 1: Agarose gel electrophoresis analysis for the *nuc* gene in *Staphylococcus aureus* isolates. Lane 17, molecular size markers; lane 16, *S. aureus* PTCC 1112 (positive control); lanes 1-15 *S. aureus* isolates. Arrow indicates the 255 bp amplicon

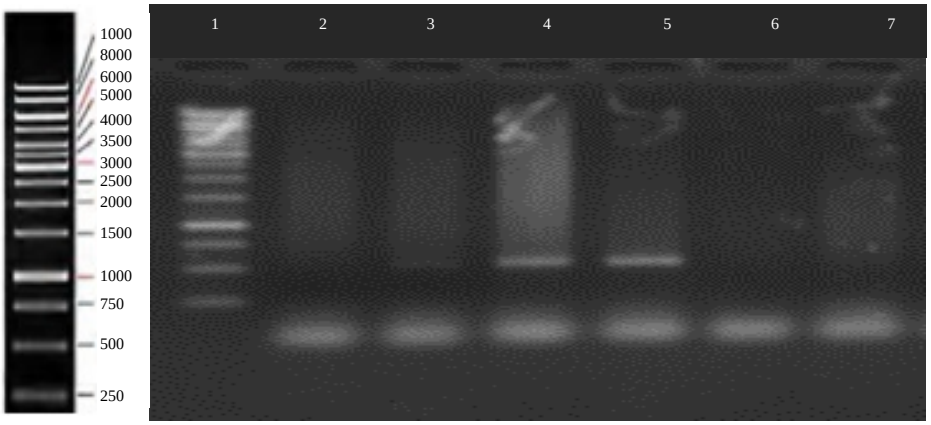

Fig. 2: Agarose gel electrophoresis analysis for the *mec-A* gene in *Staphylococcus aureus* isolates. Lane 1, molecular size markers; lanes 2, 3, 4, 5, 6 and 7 *S. aureus* isolates. Arrow indicates the 533 bp amplicon. Presence of the 533 bp shows the *mec-A* gene existence in the isolates

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 microbic 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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REFERENCES

Akineden, O., A.A. Hassan, E. Schneider and E. Usleber, 2008. Enterotoxigenic properties of *Staphylococcus aureus* isolated from goatsâ_™ milk cheese. Int. J. Food. Microbiol., 124: 211-216.

Aragon-Alegro, L.C., E.M. Konta, K. Suzuki, M.G. Silva and A. Fernandes *et al.*, 2007. Occurrence of coagulase-positive *Staphylococcus* in various food products commercialized in Botucatu, SP, Brazil and detection of toxins from food and isolated strains. Food Control, 18: 630-634.

Boerema, J.A., R. Clemens and G. Brightwell, 2006. Evaluation of molecular methods to determine enterotoxigenic status and molecular genotype of bovine, ovine, human and food isolates of *Staphylococcus aureus*. Int. J. Food. Microbiol., 107: 192-201.

Cabral, K.G., C. Lammler, M. Zschock, H. Langoni, M.E.P. de Sa, C. Victoria and A.V. da Silva, 2004. Pheno and genotyping of *Staphylococcus aureus*, isolated from bovine milk samples from Sao Paulo State, Brazil. Can. J. Microbiol., 50: 901-909.

Chambers, H.F., 1997. Methicillin resistance in *Staphylococci*: Molecular and biochemical basis and clinical implication. Clin. Microbiol. Rev., 10: 781-791.

Cremonesia, P., M. Luzzanaa, M. Brascab, S. Morandi and R. Lodi *et al*., 2005. Development of a multiplex PCR assay for the identification of *Staphylococcus aureus* enterotoxigenic strains isolated from milk and dairy products. Mol. Cell. Probes, 19: 299-305.

De Boer, E., J.T.M. Zwartkruis-Nahuis, B. Wit, X.W. Huijsdens and A.J. de Neeling *et al*., 2009. Prevalence of methicillin-resistant Staphylococcus aureus in meat. Int. J. Food Microbiol., 134: 52-56.

De-Neeling, A.J., M.J.V.D. Broek, E.C. Spalburg, M.G.V. Santen-Verheuvel and W.D. Dam-Deisz *et al*., 2007. High prevalence of methicillin resistant *Staphylococcus aureus* in pigs. Vet. Microbiol., 122: 366-372.

Devriese, L.A., L.R. van Dammel and L. Famere, 1972. Methicillin (cloxacillin)-resistant *Staphylococcus aureus* strains isolated from bovine mastitis cases. Zentbl. Veterinarmed. Ser. B., 19: 598-605.

Iurlina, M.O. and R. Fritz, 2004. Microbiological quality of Port Salute Argentino cheese stored at two temperature treatments. Lebensm. Wiss. Technol., 37: 739-748.

Japooni, A., A. Alborzi, F. Orafa, M. Rasouli and S. Farshad, 2004. Distribution patterns of methicillin resistance genes (mecA) in *Staphylococcus aureus* isolated from clinical specimens. Iran. Biomed. J., 8: 173-178.

Jones, T.F., M.E. Kellum, S.S. Porter, M. Bell and W. Schaffner, 2002. An outbreak of community-acquired foodborne illness caused by methicillin-resistant *Staphylococcus aureus*. Emerg. Infect. Dis., 8: 82-84.

Katsuda, K., E. Hata, H. Kobayashi, M. Kohmoto, K. Kawashima, H. Tsunemitsu and M. Eguchi, 2005. Molecular typing of *Staphylococcus aureus* isolated from bovine mastitic milk on the basis of toxin genes and coagulase gene polymorphisms. Vet. Microbiol., 105: 301-305.

Kim, C.H., M. Khan, D.E. Morin, W.L. Hurley and D.N. Tripathy *et al*., 2001. Optimization of the PCR for detection of *Staphylococcus aureus nuc* gene in bovine milk. J. Dairy Sci., 84: 74-83.

Kluytmans, J., W. van Leeuwen, W. Goessens, R. Hollis and S. Meser *et al*., 1995. Food-initiated outbreak of methicillin-resistant *Staphylococcus aureus* analyzed by pheno- and genotyping. J. Clin. Microbiol., 33: 1121-1128.

Mahony, R., Y. Abbott, F.C. Leonard, B.K. Markey and P.J. Quinn *et al*., 2005. Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from animals and veterinary personnel in Ireland. Vet. Microbiol., 109: 285-296.

Merlino, J., J. Watson, B. Rose, M. Beard-Pegler, T. Gottlieb, R. Bradbury and C. Harbour, 2002. Detection and expression of methicillin/oxacillin resistance in multidrug-resistant and non-multidrug-resistant *Staphylococcus aureus* in Central Sydney, Australia. J. Antimicrob. Chemother., 49: 793-801.

Nafisi, M.R., H. Kalhor, B. Zamanzad, A. Karimi, A. Farokhi and M. Validi, 2008. Comparison of agar screen and duplex-PCR in determination of methicillin resistant *Staphylococcus aureus* (MRSA) strains isolated from nose of personnel in Hajar hospital of Shahre-kord. Arak Univ. Med. Sci. J., 11: 94-101.

Normanno, G., G. La Salandra, A. Dambrosio, N.C. Quaglia and M. Corrente *et al*., 2007. Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. Int. J. Food Microbiol., 115: 290-296.

O'Brien, M., K. Hunt, S. McSweeney and K. Jordan, 2009. Occurrence of foodborne pathogens in Irish farmhouse cheese. Food Microbiol., 26: 910-914.

Pereira, V., C. Lopes, A. Castro, J. Silva, P. Gibbs and P. Teixeira, 2009. Characterization for enterotoxin production, Virulence factors, and antibiotic susceptibility of *Staphylococcus aureus* isolates from various foods in Portugal. Food Microbiol., 26: 278-282.

Rahimi, F., M. Bouzari, J. Vandyousefi, Z. Maleki, S.S. Kashani and S. Davoudi, 2008. Analysis of antibiotic resistance and detection of mecA gene in *Staphylococcus aureus* isolated from hospitals and medical laboratories in Tehran. Iran. Biol. J., 21: 64-74.

Saei, H.D., M. Ahmadi, K. Mardani and R.A. Batavani, 2009. Molecular typing of*Staphylococcus aureus* isdated from bovine mastitis based on polymorphism of the coagulase gene in the north west of Iran. Vet. Microbiol., 137: 202-206.

Scherrer, D., S. Corti, J.E. Muehlherr, C. Zweifel and R. Stephan, 2004. Phenotypic and genotypic characteristics of *Staphylococcus aureus* isolates from raw bulk-tank milk samples of goats and sheep. Vet. Micriobiol., 101: 101-107.

Tamagnini, L.M., G.B. Sousa, R.D. Gonzalez and C.E. Bude, 2006. Microbiological characteristics of Crottin goat cheese made in different seasons. Small Rumin. Res., 66: 175-180.

Zamani, A., S. Sadeghian, J. Ghaderkhani, M.Y. Alikhani and M. Najafimosleh *et al*., 2007. Detection of methicillin-resistance (mec-A) gene in *Staphylococcus aureus* strains by PCR and determination of antibiotic susceptibility. Ann. Microbiol., 57: 273-276.