Iranian Journal of Microbiology
Volume 6 Number 5 (October 2014) 345-349

ORIGINAL ARTICLE

Prevalence of enterotoxigenic *Staphylococcus aureus* in organic milk and cheese in Tabriz, Iran

Yalda Rahbar Saadat[1], Abbas Ali Imani Fooladi[2], Reza Shapouri[1], Mir Mohsen Hosseini[1], Zahra Deilami Khiabani[1*]

[1]Department of Microbiology, Faculty of Basic and Medical Science, Islamic Azad University, Zanjan Branch, Zanjan, Iran.[2]Applied Microbiology Research Center, Baqiyatallah University Medical of Sciences, Tehran, Iran.

Received: June 2014, **Accepted:** September 2014

ABSTRACT

Background and Objectives: Staphylococcal food poisoning is a gastrointestinal disease, which is caused by consumption of contaminated food with enterotoxins produced by *Staphylococcus aureus* (SEs). Milk and its products are known sources of food borne diseases. This study was carried out to evaluate the prevalence of enterotoxigenic *S. aureus* strains in organic milk and cheese in Tabriz - Iran.

Materials and Methods: A total of 200 samples (100 milk samples and 100 cheese samples) were collected from farms and milk collection points in Tabriz - Iran. The samples were cultured and identified by standard bacteriological methods, then PCR was performed to detect *sea* gene.

Results and Conclusion: *Staphylococcus aureus* was found in 27% of all samples (milk and cheese). Results of PCR showed that 12.96% of *S. aureus* isolates possessed *sea* gene. It suggested the potential public health threat of *S. aureus* resulting from contamination of dairy products. So, efforts are required to improve safety standards for preventing staphylococcal food poisoning.

Keywords: *Staphylococcus aureus*, Enterotoxin, Organic Milk, Cheese.

INTRODUCTION

Staphylococcus aureus is an important food-borne pathogen involved in a variety of invasive diseases (1). It produces many toxins as well as non-toxic enzymes and can facilitate bacterial attack and proliferation in the body of host (2). Some strains produce staphylococcal enterotoxins (SEs) that can cause food poisoning if food containing preformed SE is ingested. Symptoms of staphylococcal food poisoning (SFP) have a rapid onset (2 to 6 h) and may include vomiting, stomach pain and diarrhea (3). Enterotoxins from *S. aureus* strains can be classified into 18 serological types: A-U (except S, F and T)(2,4-9). The SEs are a group of heat stable and pepsin resistant exotoxins encoded by genes in the chromosome, pathogenicity island, phages or plasmids (2). For production of sufficient amount of toxin to cause intoxication symptoms, 10^5 CFU/ml or CFU/g enterotoxigenic strains of bacteria are needed (10). SEs are low molecular weight proteins (MW 26.900 – 29600 KD) (11). SEA is a leading cause of food intoxication (12) and is an extremely potent gastrointestinal toxin, as little as 100 ng is sufficient to cause symptoms of intoxication (13).

Milk and milk products have frequently been implicated in staphylococcal food poisoning and contaminated raw milk is often involved (2, 3). *S. aureus* mastitis is a serious problem in dairy

*Corresponding author: Zahra Deilami Khiabani PhD. Address: Department of Microbiology, Faculty of Basic and Medical Science, Islamic Azad University, Zanjan Branch, Zanjan, Iran.
Tel: +98-241-4221030
Fax: +98-241-4220030
E-mail: zdeilami@yahoo.com

Table 1. Primers for the detection of *S. aureus nuc* and *sea* genes

Primer name	Nucleotide sequence	Product size	Reference
Nuc F	5'-GCGATTGATGGTGATACGGTT-3'	279 bp	22
Nuc R	5'-AGCCAAGCCTTGACGAACTAAAGC-3'	279 bp	
Sea F	5'-TTGCGTAAAAAGTCTGAATT-3'	552 bp	23
Sea R	5'-ATTAACCGAAGGTTCTGTAGA-3'	552 bp	

production and infected animals may contaminate bulk milk. Human handlers, milking equipment, the environment and the udder and teat skin of dairy animals are other possible sources of bulk milk contamination (3, 14). There are several methods for detection of enterotoxigenic bacteria. PCR is recommended for detection of *S. aureus* enterotoxin genes (15).

Surveys to detect classical enterotoxins and to identify enterotoxin genes in *S. aureus* from milk and milk products have been conducted in many counties including Italy, Norway, Turkey, Brazil and Iran (Tehran) (16-20). However, there are no published reports about presence of SEA gene in milk and its products in Tabriz-Iran. Therefore, this study was conducted to investigate the presence of SEA gene of *S. aureus* in organic milk and cheese using PCR method in Tabriz-Iran.

MATERIALS AND METHODS

Specimen collection. A total of 200 samples (100 milk samples, 100 cheese samples) were collected from milk collection points and food stores in Tabriz, Iran. The samples were collected in sterile containers, immediately kept in an ice box and transported to the laboratory.

Microbiological analysis. The samples were investigated microbiologically for the presence of *S. aureus*, according to the standard method No. 6806-1 of the Institute of Standards and Industrial Research of Iran (ISIRI). The milk and cheese samples were diluted in ringer and pepton water, respectively. From each diluted solutions produced, 1 ml was transferred to Brain- Heart Infusion broth and incubated at 37^{o}C for 24 h. Baird- Parker agar supplemented with potassium tellurite and egg yolk emulsion was used for isolation of *S. aureus.* After 24-48 h incubation at 37^{o}C, the BPA plates were analyzed for the presence of *S. aureus*, which appeared as black colonies with transparent zone. Biochemical confirmation tests (Gram staining, coagulase, catalase, oxidase, VP and growth on mannitol salt agar) were carried out for final identification of the suspected *S. aureus* isolates.

DNA extraction and PCR experiments. For DNA isolation, biochemically confirmed single colonies of *S. aureus* isolates on BPA were cultured on LB broth. DNA extraction procedure was done by a commercial kit (Cinna pure kit), according to the supplier's instructions on the bacterial cells pellet from an overnight culture in LB broth. The primers used for the detection of *nuc* and *sea* genes are listed in Table 1.

Final confirmation of the strains was carried out using PCR with *nuc* gene. PCR reactions were performed in reaction buffer (10x), $MgCl_2$ (50mM) in a total volume of 25 µl, containing 5 µl of template DNA, 0.75 µl of each primers (10 pm), 0.25 µl of dNTP (10 mM), 0.2 µl of Taq DNA polymerase (5 unit/µl) and 14.05 µl of ddH_2O. PCR was performed under the following conditions: initial denaturation at 94^{o}C for 5 min, subsequently followed by 30 cycles of 94^{o}C for 1 min, 55^{o}C for 1 min and 72^{o}C for 1 min with a final extention of 5 min at 72^{o}C. The amplified products were shown by electrophoresis on 1% gel agarose containing ethidium bromide. Gels were viewed by UV Transillumination and photographed (Fig. 1).

After final confirmation of the isolates, PCR reaction was performed for *sea* gene. PCR reaction was conducted like the previous test but with some changes in annealing temperature (50^{o}C for 1 min). The presence of 552 bp amplicon indicates the positive samples for *sea* gene in these isolates.

RESULTS AND CONCLUSION

In the present study, *S. aureus* was observed in 54 (27 %) out of 200 samples: 9 from milk and 45 from

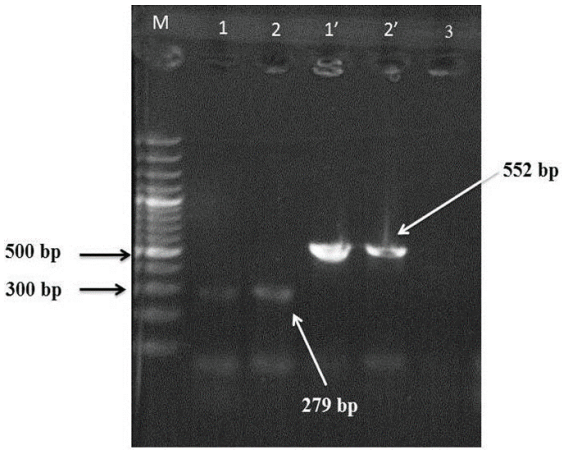

Fig. 1. 1% Agarose electrophoresis patterns showing PCR products. Lane M, standard molecular size marker (100 bp).
Lanes 1, 1': positive control.
Lanes 2, 2': *nuc* and *sea* positive isolate.
Lanes 3: negative control.

cheese samples. Total *S. aureus* counts determined between 10^2 and 10^6 CFU/ml in samples. The number of bacteria in contaminated samples by CFU/ml is shown in Table 2. For further molecular confirmation of *S. aureus* colonies, PCR reaction for *nuc* gene was performed. All 54 isolates carried *nuc* gene. DNA from those isolates was examined for the presence of *sea* gene. Results showed that 7 out of 54 *S. aureus* isolates harbored *sea* gene (Table 2).

Several researchers have reported different results of the number of bacteria in contaminated dairy products by CFU/ml (10, 20, 21). Variations in *S. aureus* count in dairy products may depend on sanitary precautions during milk processing chain. The existence of *S. aureus* in food and dairy products was previously confirmed by Imani Fooladi in Iran (20). SEA is the most common enterotoxin recovered from food poisoning outbreaks in the USA (77.8% of all outbreaks)(24).

In the current study, we used Baird- Parker agar for isolation of *S. aureus* from dairy products. This method was also employed by Boerema *et al.* (25), Rall *et al.* (26), and Ertas *et al.* (27), in same products. Considering the findings of Brakstad *et al.*(22), and comparing them to our study, it can be stated that the PCR for amplification of the *nuc* gene has potential for rapid diagnosis and confirmation of *S. aureus* isolates. The present study aimed to evaluate the prevalence rate of *sea* gene in organic milk and cheese in Tabriz, Iran. Outside of Iran, various results have been reported on the presence of *S. aureus* and its enterotoxins in milk and its products (28-31).

In the current study, we found that 27% of the organic milk and cheese samples were contaminated by *S. aureus*. PCR results for *sea* gene, showed that 12.96% of the isolates possessed *sea* gene. Similar level of incidence was reported by Holeckova *et al.,* (32), Imani Fooladi *et al.,* (20) and El-Jakee *et al.* (33). In contrast to the above results, Ertas *et al.* (27) reported lower incidence of *sea* gene (1.6%). This discrepancy could be attributed to the improvements in the handling and sanitary procedures during milking and its processing. It was concluded that even though the level of microorganisms in dairy products was not sufficient to cause disease, the presence of toxins could be considered a potential risk for public health.

Several conditions such as delay in processing, inadequate refrigeration, poor personal hygiene and post process contamination are associated with staphylococcal growth and enterotoxin production (34). Therefore it is essential to ensure high safety standards for preventing staphylococcal food poisoning. In conclusion, the results of this study provides some important preliminary data about the prevalence of enterotoxigenic *S. aureus* in organic milk and cheese in Tabriz, Iran. Consumption of organic milk and specially cheese is still widespread and could cause a potential risk to public health, so an effective reduction of contamination levels could be achieved by improving sanitation and hygiene procedures. Further investigations are needed to evaluate the production of other toxins in milk and cheese.

Table 2 : Prevalence of *sea* gene in *S. aureus* isolated from organic milk and cheese.

Types of samples	Sample size	No. of *S. aureus* isolates	Distribution CFU/ml 10^2-10^4	10^4-10^6	No. of enterotoxigenic *S. aureus* (*sea gene*)
Milk	100	9	6(66.6%)	3(33.3%)	2
Cheese	100	45	26(57.7%)	19(42.2%)	5
Total	200	54(27%)	-	-	7 (12.96%)

REFERENCES

1. Morandi S, Brasca M, Andrighetto C, Lombardi A, Lodi R. Phenotypic and genotypic characterization of *Staphylococcus aureus* strains from Italian dairy products. *Int J Microbiology* 2009; 1-7.
2. Dinges M, Orwin P, Schlievert P. Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Rev* 2000; 13:16-34.
3. Jørgensen HJ, Mørk T, Caugant DA, Kearns A., Rørvik LM. Genetic Variation among *Staphylococcus aureus* Strains from Norwegian Bulk Milk. *Appl Environ Microbiol* 2005;71:8352-8361.
4. Holtfreter S, Broker BM, Staphylococcal superantigens: Do they play a role in sepsis?. *Arch Immunol Ther Exp* 2005;53:13-27.
5. Letertre, C, Perelle S, Dilasser F, Fach P. Identification of a new putative enterotoxin SEU encoded by the egc cluster of *Staphylococcus aureus*. *J Appl Microbiol* 2003; 95:38–43.
6. Omoe, K, Hu DL, Takahashi-Omoe H, Nakane A, Shinagawa K. Identification and characterization of a new staphylococcal enterotoxin-related putative toxin encoded by two kinds of plasmids. *Infect Immun* 2003; 71:6088–6094.
7. Orwin, PM, Fitzgerald JR, Leung DY, Gutierrez JA, Bohach GA, Schlievert P M. Characterization of *Staphylococcus aureus* enterotoxin. *Infect Immun* 2003; 71: 2916–2919.
8. Orwin PM, Leung DY, Donahue HL, Novick RP, Schlievert PM. Biochemical and biological properties of staphylococcal enterotoxin K. *Infect Immun* 2001; 69:360–366.
9. Orwin PM, Leung DY, Tripp TJ, Bohach GA, Earhart CA, Ohlendorf DH, et al. Characterization of a novel staphylococcal enterotoxin-like superantigen, a member of the group V subfamily of pyrogenic toxins. *Biochemistry* 2002; 41:14033–14040.
10. Arcuri EF, Angelo FF, Guimarães MF, Talon R, Borges Mde F, Leroy S, et al. Toxigenic status of *staphylococcus aureus* isolated from bovine raw milk and minas frescal cheese in Brazil. J Food Protect 2010;73: 2225-2231.
11. Martin MC, Fueyo JM, Gonzales-Hevia JM, Mendoza MC. Genetic procedure for identification of enterotoxigenic strains of *Staphylococcus aureus* from three food poisoning outbreaks. *J Food Microbiol* 2004; 94:279-286.
12. Archer DL, Young FE. Contemporary issues: disease with a food vector. *ClinMicrobiol Rev* 1988;1: 377–398.
13. Evenson, MI., Hinds MW, Bernstein RS, Bergdoll MS. Estimation of human dose of staphylococcal enterotoxin A from a large outbreak of staphylococcal food poisoning involving chocolate milk. *Int J Food Microbiol* 1988; 7:311–316.
14. Zadoks RN, van Leeuwen WB, Kreft D, Fox LK, Barkema HW, Schukken YH, et al. Comparison of *Staphylococcus aureus* isolates from bovine and human skin, milking equipment, and bovine milk by phage typing, pulsed-field gel electrophoresis, and binary typing. *J Clin Microbiol* 2002; 40:3894–3902.
15. Van Belkum A. Molecular diagnostics in medical microbiology: Yesterday, today and tomorrow. *Curr Opin Pharmacol* 2003; 3: 497–501.
16. Morandi S, Brasca M, Lodi R, Cremonesi P, Castiglioni B. Detection of classical enterotoxins and identification of enterotoxin genes in *Staphylococcus aureus* from milk and dairy products. *Vet Microbiol* 2007; 124: 66-72.
17. Jorgensen H J, Mork T, Hogasen H R, Rovik L M. Enterotoxigenic *Staphylococcus aureus* in bulk milk in Norway. *J Appl Microbiol* 2005; 99: 158-167.
18. Boynukara B, Gulhan T, Alisarli M, Gurturk K, Solmaz H. Classical enterotoxigenic characteristics of *Staphylococcus aureus* strains isolated from bovine subclinical mastitis in Van, Turkey. *Int J Food Microbiol* 2008; 125: 209-211.
19. Rall VLM, Vieira FP, Rall R, VieitisRL, Fernades Jr, Candeias JMG, et al. PCR detection of staphylococcal enterotoxin genes in *Staphylococcus aureus* strains isolated from raw and pasteurized milk. *Vet Microbiol* 2008; 132: 408-413.
20. ImaniFooladi AA, Tavakoli HR, Naderi A. Detection of enterotoxigenic *Staphylococcus aureus* isolates in domestic dairy products, *Iran J Microbial* 2010; 2: 137-142.
21. Gucukoglu A, OnurKevenek T, Uyanik T, Cadirci O, Terzi G, Alisarli M. Detection of enterotoxigenic *Staphylococcus aureus* in raw milk and dairy products by multiplex PCR. *J Food Sci* 2012;77: M620-M623.
22. Brakstad O.G, Aasbak k, Maeland J. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* Gene. *J Clin Microbiol* 1992; 30: 1654-1660.
23. Saadati M, Barati B, Doroudian M, Shirzad H, Hashemi M, Hosseini SM, et al. Detection of Sea, Seb, Sec, Seq genes in *staphylococcus aureus* isolated from nasal carriers in Tehran province, Iran; by multiplex PCR. *JPS* 2011; 2: 34-40.
24. Balabana N, Rasooly A. Staphylococcal enterotoxins. *Int J Food Microbiol* 2000; 61: 1-10.
25. Boerema J A, Clemens R, Brightwell G. 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* 2006; 107: 192-201.
26. Rall VL, Vieira FP, Rall R, Vieitis RL, Fernandes A , Candeias JM. PCR detection of staphylococcal enterotoxin genes in *Staphylococcus aureus* strains isolated from raw and pasteurized milk. *Vet Microbiol* 2008; 132: 408-413.
27. Ertas N, Gonulalan Z, Yildirim Y, Kum E. Detection of *Staphylococcus aureus* enterotoxins in sheep cheese

and dairy desserts by multiplex PCR technique. *Int J Food Microbiol* 2010; 15(142):74-77.
28. De Buyser ML, Dufour B, Maire M, Lafarge V. Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *Int J Food Microbiol* 2001; 67:1–17.
29. Kousta M, Mataragas M, Skandamis P, Drosinos EH. Prevalence and sources of cheese contamination with pathogens at farm and processing levels. *Food Control* 2010;21: 805-815.
30. Kérouanton A, Hennekinne JA, Letertre C, Petit L, Chesneau O, Brisabois A, et al. Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. *Int J Food Microbiol* 2007; 115: 369-375.
31. Bingol E, Çetin O, Çolak H, Hampikyan H. Presence of enterotoxin and verotoxin in Turkish cheeses sold in İstanbul. *Turk J Vet Anim Sci* 2012; 36: 424-432.
32. HoieckovaB, Kalinacova V, Gondol J, Fotta M, Holoda E. Production of enterotoxins by *Staphylococcus aureus* isolated from sheep milk. *Bull Vet Inst* 2004; 48: 41-45.
33. El-Jakee J, Zaki E, Randa F. Properties of enterotoxigenic *S. aureus* isolated from mastitic cattle and buffaloes in Egypt. *J Am Sci* 2010; 6:170-178.
34. Wieneke A, Roberts D, Gilbert R. Staphylococcal food poisoning in the united kingdom,1969-90. *Epidemiol Infect* 1993; 110: 519-531.