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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

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Selective Amplification of *SEA, SEB* and *SEC* Genes by Multiplex PCR for Rapid Detection of *Staphylococcus aureus*

Hamed Ahari[1], Delavar Shahbazzadeh[2]* and Ali Misaghi[3]
[1]Veterinary Section, Nanonasb Co, Member of YRC., Tehran, Iran
[2]Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran
[3]Food Hygiene Group, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

Abstract: *Staphylococcus spp.*, is a common bacteria in cattle mastitis. Detection of the low number of bacteria requires a sensitive method. We used Multiplex Polymerase Chain Reaction (M-PCR) assay to detect the genes encoding SEA, SEB, SEC toxins that are specific in *Staphylococcus aureus* bacteria. The gene of 23S rRNA that is conserved in all *Staphylococcus spp.*, also used as control. Due to the stiffness and low effective enzyme activity such as lyzozyme, proteinase k and mutilysine on bacterial cell wall, it lysed with liquid nitrogen and multiplex PCR was performed after DNA extraction. The obtained results in molecular method were compared with cell culture. The total number of 60 samples of non pasteurized milk was collected in Tehran province restrict from dairy cattle housing and 4 positive samples were detected by PCR and microbial cell culture methods. The accuracy of the test was monitored by using serial dilution (1-I0^6) of overnight cell culture of *Staphylococcus spp.*, bacteria (OD600:0.02 = 10^7 cell). It showed that the sensitivity of PCR is 10 bacteria per ml of cells within few hours, meanwhile in bacterial blood agar cell culture; the number of 100 cells was detected after 48 h. This is the first report of molecular detection technique over *Staphylococcus aureus* bacteria in Iran which indicates the more sensitive and rapid test that can be substituted by conventional bacterial culture method.

Key words: *Staphylococcus aureus*, PCR, 23S rRNA, staphylococcal enterotoxins

INTRODUCTION

There are many reports on food-born diseases due to the contamination with *Salmonella*, *Shigella*, *Listeria monocytogenesis* and *Staphylococcus spp.* (Bergdoll, 1990). The *Staphylococcus aureus* bacterium is the most important one. *Staphylococcus aureus* is gram-positive cocci, which causes a variety infectious in human and animals. Due to the resistance of human strains to common antibiotics, rapid diagnosis of the pathogen is critical (Da Silva *et al*., 2004; Cardoso *et al*., 1999; Chen *et al*., 2001; Da Silva *et al*., 2004). Because of widespread contamination of dairy products by *staphylococcus spp.* and low efficacy of ELISA and culture methods, PCR method is going to be substituted with the mentioned methods in rapid and accurate detection of pathogenic agents and is used as a sensitive and accurate method in rapid and proper detection of microbial infections (Mehrotra *et al*., 2000; Robbins *et al*., 1974; Stephan *et al*., 2001).

At the present work, PCR method was used to detect *Staphylococcus aureus* in non pasteurized milk samples that were collected from Tehran province restricts. The gene of 23S rRNA that is conserved in all Staphylococcus spp. and the genes encoding SEA, SEB and SEC toxins and are unique in *Staphylococcus aureus*, used for detection of the contamination by *Staphylococcus aureus*. The result obtained by multiplex PCR was compared with bacterial cell culture.

MATERIALS AND METHODS

Samples: A total of sixty raw milk samples were obtained from industrial dairy herds or directly from factory milk stored tanks located in Tehran province districts. The samples were transported on ice to the lab within 2 h, frozen and maintained at -20°C until analysis for microbial experiments. Sampling was done in certain intervals of year with correct sampling principles.

Bacterial culture and enrichment: Before molecular analysis milk samples were enriched. One milliliter of milk was added with 1 ml of brain heart infusion broth (Merck), mixed and incubated overnight at 37°C. After incubation, 1 ml of enriched sample was used for isolation of bacterial DNA directly from milk using the method described by Gillespie and Oliver (Gillespie and Oliver, 2005). Conventional bacterial methods were conducted on the milk samples to identify bacteria and to determine the number of colony forming units per milliliter. Results were compared to determine the sensitivity of the multiplex PCR assay to identify bacteria from milk samples of enriched and nonenriched samples.

DNA extraction: For isolation of bacterial DNA, 1.5 ml of enriched sample was transferred to microcentrifuge tube and bacterial cells were pelleted by centrifugation

at 3,000×g for 5 min at 4°C and the fat layer and aqueous phase discarded. The amount of 30 µL of SDS %10 was added to the bacterial cells and transferred to sterile mortar that contains Liquid Nitrogen (LN) with enough volume. After freezing the cells, were squeezed by formation of crystals. Bacterial cell wall was smashed with pestle mechanical strikes and disrupted without using any lytic enzymes as the method described before for the extraction of fungal DNA (Kabir *et al*., 2003). After transferring the lysed cells to the microtube, Phenol-chloroform was added and centrifuged in 10,000×g for 10 min, supernatant (phenol) was removed and chloroform and isoamyl alcohol (1:24) was added and centrifuged at 10,000×g for 2 min and 20 µl RNase was added. The amount of 7.5 µl NaCl for precipitation of DNA and 600 µl isopropanol was added and kept in Room Temperature (RT) for 10 min. Centrifuged at 10,000×g for 10 min at 4°C, supernatant removed and the precipitation which contains DNA washed two times with 70% of ethanol and dissolved in distilled H_2O.

Molecular detection of 23S rRNA and sea_b_c genes: In the present study, the amplification parameters and primer sequences described by da Silva *et al.* and Cremonesi *et al.* [Da Silva *et al*., 2004; Cremonesia *et al*., 2005) were used (Table 1). The amplification of genes was carried out with the following thermal cycling profile: an initial denaturation at 94°C for 5 min was followed by 30 cycles of amplification (denaturation at 94°C -2 min, annealing at 57°C -1 min and extension at 72°C -1 min), ending with a final extension at 72°C for 4 min. The PCR products were separated at 3.5% agarose gel, stained with ethidium bromide and photographed under UV illumination.

Sensitivity and detection limits of PCR and pure culture: Overnigt culture of *Staph. aureus* (RTCC 2411) was used to prepare serial dilutions in Baird Parker agar (Merck), with bacterial concentrations ranging from 1-10^7 cfu/ml. DNA extraction was done to perform PCR and simultaneously, microbial culture of milk samples on Trypticase Soy Broth (TSB, Merck) was done to compare the sensitivity of molecular and culture methods.

Bacterial culture: Milk samples were centrifuged at 3,000×g for 10 min. A loopful of bacterial pellet and cream cultured on blood agar. Plates were incubated at 37°C and bacterial growth was observed at 24-h intervals for 3 d. Bacteria on primary culture medium were identified tentatively according to colony morphologic features and hemolytic characteristics. Isolates identified presumptively as staphylococci were determined using the conventional tube test with rabbit plasma (*clavo Diagnostic*_Coagulase Siena:62021/08/01). Briefly, one positive colony was transferred to 300 µl of BHI broth, from which a loopful of inoculated medium was plated in trypticase soy agar and both liquid and solid medium were incubated at 37°C for 24 -48 h. Five hundred microliter of rabbit plasma was added to BHI broth and assayed for coagulase test after incubation at 37°C for 6 h. Only complete clotted tubes were considered as positive. The suspect tubes were incubated for another 18-48 h for accurate detection.

Bacterial colony count: The amount of 0.1 ml of diluted raw milk samples of 10^{-1}-10^{-2} diluted in Ringer solution were cultured in Baird Parker Infusion agar. Total bacterial colony counts were done after incubation at 35°C for 24 h.

Calculation of kappa coefficient: The calculation was assessed by using kappa statistic formula as follow: Kappa= 0P-EP/1-EP, where OP is the observed proportion of agreement and EP is the expected proportion of agreement by chance. The kappa coefficient up to 1 is for complete agreement (Altman, 1991).

RESULTS

Uniplex PCR with each individual primer pair resulted in the amplification of single products when DNA from reference strain was used as a template. The sizes of the products obtained from control strains in both PCR designs corresponded to the predicted sizes (Fig. 1). After doing PCR reaction on standard staphylococcus strain with the four-pair primers, the specificity of these primers for this strain was proved.

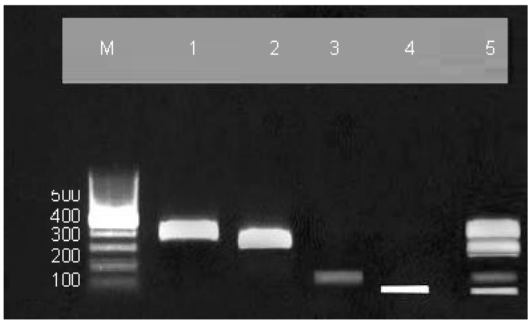

Fig. 1: Detection of *Staphylococcus aureus* genes by; uniplex PCR: Lanes 1-4: 23S rRNA gene (499 bp); SEC gene (451 bp); SEB gene (1640; SEA gene (102) and multiplex PCR Lane 5. M: 100 bp DNA ladder (Cinnagen)

In this study by serial dilution of bacterial cell culture, the sensitivity of PCR was tested on DNA extracted from standard isolate. The optimum sensitivity of the test was detected with 10 bacterial cells (Fig. 2). Meanwhile, the sensitivity of microbial culture on Baird Parker broth showed that, the dilution up to 100 bacteria is detectable after 48 h.

Table 1: Primer sequences, predicted sizes of PCR product for the amplification of *Staph. aureus* target genes

Reference	Target gene	Amplification size (bp)	Oligonucleotide sequence (30-50)	Primer Name
Cremonesi *et al.* (2005)	23S rRNA	499	TTT-GG-TCC-TTG-TCC-GGA-TGT-AGC	23S1200-F
			AGA-AT- CTT- CAC- GCT-CTC- TC	23S1698-R
da Silva *et al.* (2005)	sea	102	GGT-TAT-CAA-TGT-GCG-GGT-GG	A1
			CGG-CAC-TTT-TTT-CTC-TTC-GG	A2
da Silva *et al.* (2005)	seb	164	GTA-TGG-TGG-TGT-ACC-TGA-GC	B1
			CCA-AAT-AGT-GAC-GAG-TTA-GG	B2
da Silva *et al.* (2005)	sec	451	AGA-TGA-AGT-TGA-TGT-GTA-TGG	C1
			CAC-ACT-TTT-AGA-ATC-AAC-CG	C2

Table 2: Raw milk bacterial colony counts in positive m-PCR samples

Colony Count (cfu/ml)	Milk sample
6×10^1	1
2×10^2	2
1×10^2	3
1.6×10^2	4

In the multiplex, the amplification of one band in 23S rRNA and three bands in sea, seb and sec were successfully obtained in standard *Staph. aureus* strain (Fig. 1, lane 5). Among the 60 sixty raw milk samples analyzed by m-PCR, five were positive for some of the SEs genes investigated (Fig. 3). The last one was detected negative in cell culture but was recognized very weakly in PCR. Total bacterial counting was done at 5 positive samples obtained in m-PCR. As shown in Table 3, high bacterial colony counts was obtained in sample 4 with 2×10^2 cfu/ml and the low counting shown in sample 5 with 5×10^1 cfu/ml.

Kappa statistic was calculated to compare the observed and expected proportions on the following table of frequencies:

Table 3: Kappa number

Culture/Multi Plex PCR	Positive	Negative	Total
Positive	4 = a	1 = b	5
Negative	0 = c	55 = d	55
Total	4	60	60

Kappa = 0P-EP/1-EP
OP (Observation Proportion) = a+d/Number of samples
4+ 55/60 = 0.983 ⟶ OP = 0.983
EP (Expected Proportion) = (a+b/n)x(a+c/n)+(c+d/n)x(b+d/n)
EP = (5/60x4/60)+(55/60x56/60) = 0.855 ⟶ EP = 0.855
Kappa = 0.983-0.855/1-0.855 = 0.128/0.145 ⟶ Kappa = 0.83

DISCUSSION

Numerous methods for isolating bacterial DNA directly from milk have been reported in the literature and involve a wide variety of substances including Chelex-100 (Kim *et al.*, 2001), spin columns (Phuektes *et al.*, 2001; Phuektes *et al.*, 2003; Riffon *et al.*, 2001), lysozyme and proteinase K (Meiri-Bendek *et al.*, 2002), diatomaceous earth [Martinez *et al.*, 2001), alkaline extraction [Daly *et al.*, 2002) and pronase [Allmann *et al.*, 1995; Hein *et al.*, 2001). In this study for the first time liquid nitrogen method was employed for isolating bacterial genome directly from milk. The method is cost effective and more convenient without using any lytic enzymes. An enrichment step was added to overcome PCR inhibition and to increase the sensitivity of the assay. With this added step, sufficient bacteria were present to allow detection of as few as 1 cfu/ml [Gillespie and Oliver, 2005). The coagulase test represents the standard method for the identification of *S. aureus* in dairy products. However, an overnight incubation is necessary to obtain reliable results and this represents a strong drawback for wide diagnostic applications (Stephan *et al.*, 2001). Moreover, in staphylococcal food poisoning, the best identification of the *Staph. aureus* SE genes from food material is a crucial step to rapidly determine the SEs profile in investigated suspected food samples (Stephan *et al.*, 2001). Grinding in the presence of liquid N_2 (LN) [Kabir *et al.*, 2003), was followed to extract DNA from samples in this study.

Some *Stahp. aureus* strains produce one or more enterotoxigenic toxins including SEA, B and C. These SEs represent the main cause of staphylococcal food poisoning and are potential virulence factors that contribute toward mastitis pathogenesis [Stephan *et al.*, 2001). Furthermore, the SEs could be able to indicate the origin of the S. aureus strains because it was observed that a higher ratio of isolates from animals, produced SEC and those from humans produced mainly SEA (Bergdoll, 1990). In this study two isolates include sec or sea and one isolate with both genes were observed (Fig. 3).

Although there were variations with band intensity, their presence and sizes were the same. But, the band corresponding to the 23S rRNA resulted often more intensive than the others as described in other experiment (Cremonesia *et al.*, 2005).

In our work, we initially designed primer pairs for 23S rRNA gene and for all the known enterotoxin genes of sea, seb and sec. Several studies described that none of the investigated strains isolated from bovine and goat milk and dairy products harbored the gene of SEB [Ferens *et al.*, 1998; Omoe *et al.*, 2002; Sharma *et al.*, 2000; Najera-Sanchez *et al.*, 2003; Akineden *et al.*, 2001), but as it is shown in Fig. 1, the band of seb gene although weak, but was seen in the isolates. SEA, SEB

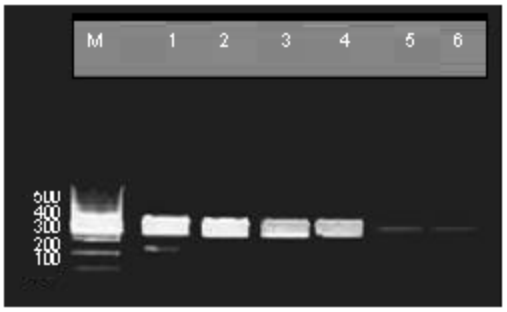

Fig. 2: PCR sensitivity test by using 23S rRNA gene (499 bp): lanes 1-6; 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 standard *Stahpylococcus aureus cells*; M, 100 bp DNA molecular marker

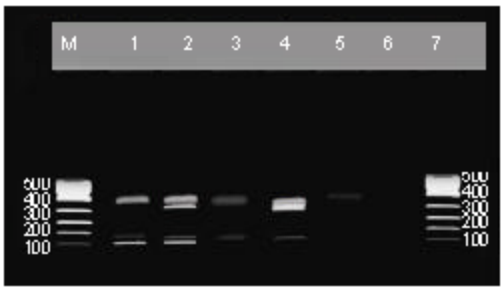

Fig. 3: Multiplex PCR amplification for the detection of *Staph. aureus* genes. Lanes 1-5, positive samples in non-pasteurized milk obtained in Tehran province districts. All samples are positive for 23S rRNA; the exact size of each band is given in Table 1; lane 6 *Staphylococcus hyicus* as negative control; M, 100 bp DNA molecular marker

and SED are the most common enterotoxins recovered from food-poisoning outbreaks in man, the significance of these novel toxins for public health is unclear (Zschock *et al*., 2005).

In addition by using bacterial culture sample 5 was false negative for *Staph. aureus* and even in coagulase test, but observed positive in m-PCR.

In our work, we analyzed 60 non-pasteurize raw milk samples from Tehran province districts, comparing our results with those obtained with microbiological methods, reveals that the m-PCR assay specificity and sensitivity makes the method effective in potentially revealing hazardous *Staph. aureus* infectious in the dairy products chain and the perfect agreement result was obtained in Kappa correlation coefficient (0.83).

Therefore, an interesting development of m-PCR assay in this study would be its application directly on milk and food samples, saving two days of analysis. The molecular method developed for the first time in Iran can be applied for rapid detection of *Staphylococcus aureus* bacteria in dairy milk samples.

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REFERENCES

Akineden, O., C. Annemuller, A.A. Hassan, C. Lammler, W. Wolter and M. Zschock, 2001. Toxin genes and other characteristics of *Staphylococcus aureus* isolates from milk of cows with mastitis. Clin. Diagn. Lab. Immunol., 8: 959-64.

Allmann, M., C. Hoefelein, E. Koppel, J. Luthy, R. Meuer, C. Niederhauser, B. Wegmuller and U. Canadian, 1995. Polymerase Chain Reaction (PCR) for detection of pathogenic microorganisms in bacteriological monitoring of dairy products. Res. Microbiol., 146: 85-97.

Altman, D.G., 1991. Practical statistics for medical research. London: Chapman and Hall, 404-9.

Bergdoll, M.S., 1990. Staphylococcal food poisoning. In: Food borne Diseases. (Diver, D.O. Ed.), Academic Press, San Diego, pp: 85-106.

Cardoso, H.F.T., N. Silva, M.J. Sena and L.S. Carmo, 1999. Production of enterotoxins and toxic shock toxin by *Staphylococcus aureus* isolated from bovine mastitis in Brazil. Lett. Appl. Microbiol., 29: 347-349.

Chen, T.-R., M.-H. Hsiao, C.-S. Chiou and H.-Y. Tsen, 2001. Development and use of PCR primers for the investigation of C1, C2 and C3 enterotoxin types of *Staphylococcus aureus* strains isolated from food-borne outbreaks. Int. J. Food Microbiol., 71: 63-70.

Cremonesia, P., M. Luzzanaa, M. Brascab, S. Morandib, R. Lodib, C. Vimercatic, D. Agnellinia, G. Caramentid, P. Moronic and B. Castiglioni, 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.

Da Silva, E.R., J.U.D. Boechat, J.C. Dias Martins, W.P. Barbosa Ferreira, A. Pimenta Siqueira and N. da Silva, 2004. Hemolysin production by *Staphylococcus* species isolated from mastitic goat milk. Small Rumin. Res., 55: 45-49.

Da Silva, E.R., L.S. do carom and N. da Silva, 2004. Detection of the enterotoxins A, B and C genes in *staphylococcus aureus* from goat and bovine mastitis in Brazilian dairy herds. Vet. Microbiol., 106: 103-107.

Daly, P., T. Collier and S. Doyle, 2002. PCR-ELISA detection of *Escherichia coli* in milk. Lett. Appl. Microbiol., 34: 222-226.

Ferens, W.A., W.C. Davis, M.J. Hamilton, Y.H. Park, C.F. Deobald, L. Fox and G. Bohach, 1998. Activation of bovine lymphocyte subpopulations by staphylococcal enterotoxin C. Infect. Immun., 66: 573-580.

Gillespie, B.E. and S.P. Oliver, 2005. Simultaneous detection of mastitis pathogens, *Staphylococcus aureus, Streptococcus uberis* and *Streptococcus agalactiae* by multiplex real-time polymerase chain reaction. J. Dairy Sci., 88: 3510-3518.

Hein, I., D. Klein, A. Lehner, A. Bubert, E. Brandl and M. Wagner, 2001. Detection and quantification of the *iap* gene of *Listeria monocytogenes* and *Listeria innocua* by a new real-time quantitative PCR assay. Res. Microbiol., 152: 37-46.

Kabir, S., Abir, N. Rajendran, T. Amemiya and K. Itoh, 2003. Quantitative measurement of fungal DNA extracted by three different methods using real-time polymerase chain reaction. J. Biosci. Bioengi., 96: 337-343.

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

Martinez, G., J. Harel and M. Gottschalk, 2001. Specific detection by PCR of *Streptococcus agalactiae* in milk. Can. J. Vet. Res., 65: 68-72.

Mehrotra, M., G. Wang and W.M. Johnson, 2000. Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1 and methicillin resistance. J. Clin. Microbiol., 38: 1032-1035.

Meiri-Bendek, I., E. Lipkin, A. Friedman, G. Leitnen, A. Saron, S. Friedman and Y. Kashi, 2002. A PCR-based method for the detection of *Streptococcus agalactiae* in milk. J. Dairy Sci., 85: 1717-1723.

Najera-Sanchez, G., R. Maldonado-Rodriguez, P.R. Olvera and L. Mota de la Garza, 2003. Development of two multiplex polymerase chain reaction for the detection of enterotoxigenic strains of Staphylococcus aureus isolated from foods. J. Food Prot., 66: 1055-62.

Omoe, K., M. Ishikawa, Y. Shimoda, D.L. Hu, S. Ueda and K. Shinagawa, 2002. Detection of seg, seh and sei genes in Staphylococcus aureus isolates and determination of enterotoxin productivities of S. aureus isolates harboring seg, seh, or sei genes. J. Clin. Microbiol., 40: 857-62.

Phuektes, P., G.R. Browning, G. Anderson and P.D. Mansell, 2003. Multiplex polymerase chain reaction as a mastitis screening test for *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae* and *Streptococcus uberis*. J. Dairy Res., 70: 149-155.

Phuektes, P., P.D. Mansell and G.F. Browning, 2001. Multiplex polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and Streptococcal causes of bovine mastitis. J. Dairy Sci., 84: 1140-48.

Riffon, R., K. Sayasith, H. Khalil, P. Dubreuil, M. Drolet and J. Lagace, 2001. Development of a rapid and sensitive test foridentification of major pathogens in bovine mastitis by PCR. J. Clin. Microbiol., 39: 2584-2589.

Robbins, R., S. Gould and M. Bergdoll, 1974. Detecting the enterotoxigenicity *of Staphylococcus aureus* strains. Appl. Microbiol., 28: 947-950.

Sharma, N.K., C.D. Rees and C.E.R. Dodd, 2000. Development of a single-reaction multiplex PCR toxin typing assay for Staphylococcus aureus strains. Appl. Environ. Microbiol., 66: 1347-53.

Stephan, R., C. Annemuller, A.A. Hassan and C.H. Lammler, 2001. Characterisation of enterotoxigenic Staphylococcus aureus strains isolated from bovine mastitis in north-east Switzerland. Vet. Microbiol., 38: 373-82.

Zschock, M., B. Kloppert, W. Wolter, H.P. Hamann and C.H. Lammler, 2005. Pattern of enterotoxin genes seg, seh, sei and sej positive *Staphylococcus aureus* isolated from bovine mastitis. Vet. Microbiol., 108: 243-249.