Detection and Identification of β-lactamase, Enterotoxin and Other Exotoxins Genes of *Staphylococcus aureus* by PCR

Y. H. Yoon* and K. I. Kim

Department of Animal Science and Technology, College of Industrial Sciences, Chung-Ang university
Ansung 456-756, Korea

ABSTRACT : *Staphylococcus aureus* is a major pathogen for cattle, causing various forms of subclinical and clinical mastitis and could be a causative agent of food poisoning, it produces various superantigenic exotoxins which have a great public health significance. A total of 72 *S. aureus* clinical isolates from dairy farms located in Kyunggi Province Korea were examined for the species identification by biochemical method, and for the detection of β-lactamase, enterotoxin and other exotoxins genes by PCR. The results of species identification by biochemical method agreed with those of PCR done with species specific primer STA-AU. β-lactamase is an enzyme closely associated with the resistance to antibiotic penicillin, which is an important means of treatment of mastitis, all the isolates were positive for the presence of genes encoding β-lactamase, which were reproduced in penicillin susceptibility disc assay. Six types of toxin genes, *Staphylococcal* enterotoxin (SE)A, SEB, SEC, SEE, toxic shock syndrome toxin (TSST-1) and exfoliative toxin A (ET A) were detected in 72 isolates by PCR associated genotypic method in this study, none of the isolates carried the genes for enterotoxin D (SED) and exfoliative toxin B (ETB). The occurrence rate of exotoxin genes rated as 12.5%, and the precision of the PCR identification results has been confirmed using the reference strains. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 3 : 425-429)***

Key Words : *Staphylococcus aureus,* Enterotoxin, PCR, β-lactamase

INTRODUCTION

Staphlyococcus aureus is one of the major pathogen of bovine mastitis and it was claimed that approximately 40% of the milk samples turned out to contain *Staphlyococcus aureus* (Larsen et al., 2002), the organism has been considered as an important organism in the public health aspects which causes food poisoning (Coia et al., 1990). Superantigenic exotoxins are produced by *S. aureus* and contains enterotoxins, exfoliative toxins and toxic shock syndrome toxins. They interfere immune system of cattle and acts as vilurence factors which are thought to contribute to the pathogenicity of the organism. It was claimed that the β-hemolytic *S. aureus* is more virulent than non-hemolytic *S. aureus*. Serological groups of enterotoxins classified as staphylococcal enterotoxin A(SEA), SEB, SEC, SED, and SEE, and SEC could be subdivided into SEC1, SEC2 and SEC3 based on the difference in the local epitopes (Johnson et al., 1991). Enterotoxins are of single chain protein structure in nature, which are resistant to the action of protein hydrolyzing enzyme pepsin, and the toxic effects could be destroyed by heat treatment at 121°C for 11minutes, however it requires 121°C 28 min for the destruction of toxic activity of SEA, SEA and SED are the types of enterotoxin frequently invloved with the outbreak of the food poisoning (Blanban and Rasooly, 2000; Fueyo et al., 2001; Alatossava et al., 2001; Onoue and Mori, 1997, Rasooly and Rasooly, 1998) β-lactamase has been known to be an enzyme associated with the resistance of penicillins, the synthesis of the protein is coded by the gene blaZ.

It is of great significance to get the rapid results of species identification and to know the penicillin resistance of the strain for the effective control of the organisms, and to get the results of toxin production capability of the strains for the precise evaluation of the endangerness of the strain if it contaminated in milk or other foods.

This study was conducted to identify rapidly the species and to determine the sensitivity against penicillin and to detect the genes of enterotoxin and other exotoxins of *S.aureus* which was isolated from the dairying environment of Korea by PCR amplification method.

MATERIALS AND METHODS

Bacterial strains and media

13 of reference strains and 72 isolates of *S. aureus* were used in this study as shown in Table 1, and they were propagated in Brain Heart Infusion broth at 37°C. Identification of the strains were carried out utilyzing API Staph System(Biomeieux, S. A. France).

Penicillin susceptibility test

Penicillin resistance test was undertaken by disc assay method, 10mg of pecicillin containing disc (Sigma, USA) was used. Staphylococcal strains were grown up to McFarland turbidity 0.5 and plated on Muller Hinton Agar and put the discs on the surface, cultivation proceeded at

* Reprint request to: Y. H. Yoon. Tel: +82-31-670-3027, Fax: +82-31-675-5216, E-mail: yungh310@post.cau.ac.kr
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Table 1. Strains and results of their PCR identification, penicillin resistance, enterotoxin and other exotoxin gene

Staphylococcus aureus	Species specific primer STAAU	Enterotoxin specific primer								β-lactamase primer *Bla Z*
		SEA	SEB	SEC	SED	SEE	TES T-1	ETA	ETE	
Reference strains										
NCTC9393	+	-	-	-	+	-	-	-	-	+
FRI913	+	+	-	+	-	+	+	-	-	+
ATCC13515	+	+	-	ND	ND	-	-	-	-	+
MNHOCH	+	-	+	ND	-	-	-	-	-	ND
MNDON	+	-	-	+	-	-	-	-	-	+
FRI472	ND	-	-	ND	+	-	-	-	-	+
FRI326	ND	-	-	-	-	+	-	-	-	+
RN4220 pMIN403	+	-	-	ND	ND	-	-	-	-	+
FRIMN8	+	+	-	ND	ND	-	+	-	-	+
RN4220	+	-	-			-	-	-	-	+
805(T)	+	-	-	+	ND	-	+	-	-	+
807(C)	+	-	-	+	ND	-	+	-	-	+
877(A-S)	+	-	-	+	ND	-	+	-	-	+
Isolates										
CU1101	ND	-	-	-	-	-	-	-	-	+
CU1102	ND	-	-	-	-	-	-	-	-	+
CU1104	+	-	-	-	-	-	-	-	-	+
CU1105	+	-	-	-	-	-	-	-	-	+
CU1106	+	-	-	-	-	-	-	-	-	+
CU1107	+	-	-	-	-	-	-	-	-	+
CU1108	+	-	-	-	-	-	-	-	-	+
CU1109	+	-	-	-	-	-	-	-	-	+
CU1110	+	-	-	-	-	-	-	-	-	+
CU1111	ND	-	-	-	-	-	-	-	-	+
CU1112	ND	-	-	-	-	-	-	-	-	+
CU1113	+	-	-	-	-	-	-	-	-	+
CU1114	+	-	-	-	-	-	-	-	+	+
CU1115	+	-	-	-	-	-	-	-	-	+
CU1116	+	-	-	-	-	-	-	-	-	+
CU1117	+	-	-	-	-	-	-	-	-	+
CU1118	+	-	-	-	-	-	-	-	-	+
CU1119	+	-	-	-	-	-	-	-	-	+
CU1121	ND	-	-	-	-	-	-	-	-	+
CU1122	+	-	-	-	-	-	-	-	-	+
CU1123	+	-	-	-	-	-	-	-	-	+
CU1125	+	-	-	-	-	-	-	-	-	+
CU1126	+	-	-	-	-	-	-	-	-	+
CU1129	+	-	-	-	-	-	-	-	-	+
CU1133	ND	-	-	-	-	-	-	-	-	+
CU1134	+	-	-	-	-	-	-	-	-	+
CU1135	+	-	-	-	-	-	+	-	-	+
CU1136	+	-	-	-	-	-	-	-	-	+
CU1137	+	-	-	-	-	-	-	-	-	+
CU1138	+	-	-	-	-	-	-	-	-	+
CU1139	+	-	-	-	-	-	-	-	-	+
CU1140	ND	-	-	-	-	-	-	-	-	+
CU1141	ND	-	-	-	-	-	-	-	-	+
CU1142	ND	-	-	-	-	-	-	-	-	+
CU1143	ND	-	-	-	-	-	-	-	-	+
CU1144	ND	-	-	-	-	-	-	-	-	+
CU1145	ND	-	-	-	-	-	-	-	-	+
CU1146	+	-	-	-	-	-	-	-	-	+
CU1147	+	-	-	-	-	-	-	-	-	+
CU1148	+	-	-	-	-	-	-	-	-	+
CU1149	ND	-	-	-	-	-	-	-	-	+
CU1150	ND	-	-	-	-	-	-	-	-	+
CU1151	+	-	-	-	-	-	-	-	-	+
CU1152	+	-	-	-	-	-	-	-	-	+
CU1154	+	-	-	-	-	-	-	-	-	+
CU1155	ND	-	-	-	-	-	-	-	-	+
CU1157	+	-	-	-	-	-	-	-	-	+
CU1158	ND	-	-	-	-	-	-	-	-	+
CU1159	+	-	-	-	-	-	-	-	-	+
CU1160	+	-	-	-	-	-	-	-	-	+
CU1162	ND	-	+	-	-	+	-	-	-	+
CU1163	ND	-	-	-	-	-	-	-	-	+
CU1164	+	-	-	-	-	-	-	-	-	+
CU1165	+	-	-	-	-	-	-	-	-	+
CU1166	+	-	-	-	-	-	-	-	-	+
CU1167	+	-	-	-	-	-	-	-	-	+
CU1169	+	-	-	-	-	-	-	-	+	+
CU1170	+	-	-	-	-	-	-	-	-	+
CU1171	ND	-	-	-	-	-	-	-	-	+
CU1172	+	-	-	-	-	-	-	-	-	+
CU1173	ND	-	-	-	-	-	-	-	-	+
CU1174	+	-	-	-	-	-	-	-	-	+
CU1175	+	-	-	-	-	-	-	-	-	+
CU1176	ND	-	+	-	-	+	-	-	-	+
CU1180	ND	+	-	-	-	-	-	-	-	+
CU1182	ND	-	-	-	-	-	-	-	-	+
CU1183	+	-	-	-	-	-	-	-	-	+
CU1185	ND	-	-	-	-	-	-	-	-	+
CU1188	+	-	-	-	-	-	-	-	-	+
CU1189	+	-	-	-	-	-	-	-	-	+
CU1190	+	-	-	-	-	-	-	-	-	+
CU1192	+	-	-	+	-	-	-	-	-	+

37°C for 16 h and results of inhibitory zone diameter were read based on the criteria for the resistance (<28 mm; resistant, >29 mm; sensitive).

Preparation of genomic DNA

Genomic DNA of *S. aureus* was prepared as following, 1.5 ml of overnight culture was centrifuged at 12,000×g to get cell pellet and washed with 50 mM EDTA, and the the genomic DNA was isolated by the genomic DNA purification kit(Promega, USA).

Detection of β-lactamase gene

For the PCR amplification of Beta-lactamase gene, primer *Bla*Z I (5'-gctcatattggtgtttatgc-3') and *Bla*Z II (5'-atcactatatgtcattgaagc-3') were used.

The concentration of *Taq* polymerase, dNTP and magnesium chloride adjusted to 1.5 mM, the thermal cycler (Perkin Elmer 2,400 USA) was maintained at 92°C for 2 min and 95°C 30 sec for denaturation, 55°C 30 sec for annealing, 72°C 30 sec for extension and cycled 30 times. The amplification products were purified by utilyzing the PCR purification kit (Biolabs USA) and the products were tested on electrophoresis system for the size determination.

PCR amplification of species specific, enterotoxin, toxic shock syndrome, and exfoliative toxin primers Primers for the amplification reaction were synthesized as the following sequences, STAA-AU I (5´-tcttcagaagatgcggaata-3´), STAA-AU II (5´-taagtcaaacgttaacatacg-3´), SEA-1 (5´-ttggaaacggttaaaacgaa-3´), SEA-2 (5´-gaaccttcccatcaaaaaca-3´), SEB1(5´tcgcatcaaactgacaaacg-3´), SEB-2 (5´-gcaggtactctataagtgcc-3´), SEC-1 (5´-gacataaaagctaggaattt-3´), SEC-2 (5´-aaatcggattaacattatcc-3´), SED-1 (5´-ctagtttggtaatgtctcct-3´), SED-2 (5´-taatgctatatcttataggg-3´), SEE-1 (5´-tagataaagttaaagcaagc-3´), SEE-2 (5´-taacttaccgtggacccttc-3´), TSST-1 (5´-atggcagcatcagcttgata-3´), TSST-2 (5´tttccaataaccacccgttt-3´), ETA-1 (5´-ctagtgcatttgttattcaa-3´), ETB-2(5´-tccatcgataatatacctaa-3´). the concentration of *Taq* polymerase, dNTP and magnesium chloride adjusted to 1.5 mM, the thermal cycler (Perkin Elmer 2,400 USA) was maintained at 92℃ for 2 min and 95℃ 30 sec for denaturation, 55℃ 30sec for annealing, 72℃ 30 sec for extension and cycled 30 times. The amplification products were purified by utilyzing the PCR purification kit (Biolabs USA) and the size of gene in the amplification products were estimated at agarose gel electrophoresis system.

Electrophoresis and imaging, A 5- µl aliquot of of the reaction mixture was combined with 2 µl of loading buffer and the preparation was electrophoresed on 1.0% agarose gel, The gels were stained with ethidium bromide and photographed on a UV transilluminator.

RESULTS AND DISCUSSION

Identification by species specific primer

The size of gene in amplification products by primer STAA-AU I (5´-tcttcagaagatgcggaata-3´) and STAA-AU II (5´-taagtcaaacgttaacatacg-3’´) were estimated as 420 bp and the results of PCR methods for the 11 reference strains agreed completely and those of 48 isolates were agreed with those of biochemical identification results as shown in Table 1. The identification results by PCR could not always successful one, possible reason for the few failures to the species level may be the variation of ribosomal RNA operons between strains.

Penicillin resistance (β– lactamase gene) of *S. aureus*

A primer for β–lactamase gene was used in the PCR amplification reaction with template DNA from reference strains and 260 bp of gene was detected in 73 isolates of *S. aureus* as shown in Figure 1 and the results indicated that all of the strains were resistant to penicillin antibiotics. The predominance of penicillin resistant strains could be due to overdose use of penicillin. Penicillin resistance is complex feature, which is affected by many factors, the resistance may be carried by plasmid or chromosomal DNA. In addition to blaZ and the genetic polymorphysm of blaZ gene itself, other penicillin genes such as genes encoding peincillin binding protein (PBP) variants may be involved (Hackbarth et al., 1995). It has been demonstrated that β-lactamase production was induced by milk in β-lactamase positive strains, much of the increase in resistance to penicillin G caused by milk can be attributed to milk fat grobules, the increase in resistance was related to the binding capacity of the bacteria to milk fat grobules as well as to capsule formation by the bacteria. (Ali-Vehmas et al., 1997). All the test strains were shown to be penicillin resistant in disc assay.

Detection of Enterotoxin, toxic shock syndrome toxin (TSST) and exfoliative genes

PCR amplification products of enterotoxin genes of reference strains of *S. aureis* are shown in Figure 1, strain ATCC 13515 was reference strain producing SEA, the amplified product size was shown to be 120 bp, which agreed with the result of Betlay et al. (1988). Strain MNHCOH was reference strain producing SEB, the product size was shown to be 490 bp. Strain MNDON, FRI472, and FRI326 showed gene of enterotoxin those of enterotoxin C, D, E respectively, and the size of gene were 250 bp, 320 bp, and 170 bp respectively, which agreed with the results of Junes and Khan (1986). The size of toxic shock syndrome

Figure 1. Agarose gel electrophoresis of PCR products for reference strains by *bla*Z I, II primer as β-lactamase gene encoding.
Lane 1; size marker, lane 2; *Streptococcus agalactiae* CU, lane 3; *Streptococcus agalactiae* ATCC 13813, lane 4; *Streptococcus uberis* CU, lane 5; *Streptococcus uberis* ATCC 27958, lane 6; *Streptococcus dysagalactiae* 27957, lane 7; *Streptococcus pyogenes* CU, lane 8; *Staphylococcus epidermidis* CU, lane 9; *Staphylococcus epidermidis* ATCC 12228, lane 10; *Staphylococcus hyicus* NVRI, lane 11; *Staphylococcus aureus* NCTC 9393, lane 12; *Staphylococcus aureus* FRI 913, lane 13; *Staphylococcus aureus* ATCC 13515, lane 14; *Staphylococcus aureus* MNHOCH, lane 15; *Staphylococcus aureus* MNDON, lane 16; *Staphylococcus aureus* FRI 472, lane 17; *Staphylococcus aureus* FRI 326, lane 18; *Staphylococcus aureus* RN4220 pMIN403, lane 19; *Staphylococcus aureus* FRI MN8, lane 20; *Staphylococcus aureus* RN4220, lane 21; *Staphylococcus aureus* 805(T), lane 22; *Staphylococcus aureus* 807(C), lane 23; *Staphylococcus aureus* 877(A-S)

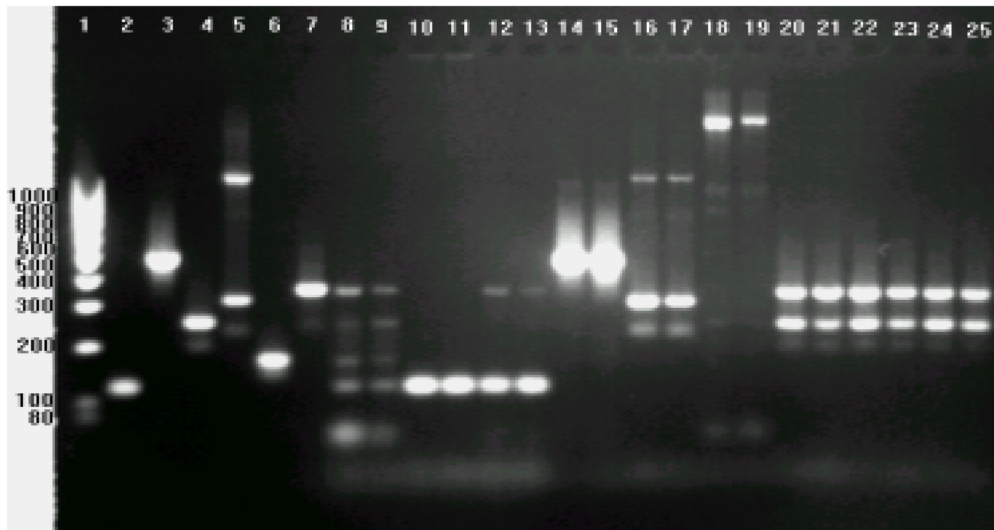

Figure 2. Agarose gel electrophoresis of PCR pruducts for reference strains by single and multiple enterotoxin specific primers.
Lane 1; 100 ladder size marker, lane 2; *S. aureus* ATCC13515, lane 3; *S. aureus* MNHOCH, lane 4; *S. aureus* MNDON, lane 5; *S. aureus* FRI472, lane 6; *S. aureus* FRI326, lane 7; *S. aureus* FRIMN8, lane 8, lane9; *S. aureus* FRI913, lane 10, lane 11; *S. aureus* ATCC13515, lane 12, lane13; *S. aureus* FRI MN8, lane 14, lane15; *S. aureus* MNHOCH, lane 16, lane 17; *S. aureus* FRI472, lane 18, lane19; *S. aureus* RN4220, lane 20, lane 21; *S. aureus* 805(T), lane 22, lane 23; *S. aureus* 807(C), lane 24, lane 25; *S. aureus* 877(A-S)

toxin (TSST) gene from was 350 bp.

Mixture of primers of SEA SEC SEE and TSST genes produced the products of PCR amplification products as shown in lane 8 and 9 in Figure 2.

A total of 72 *S. aureus* isolates from dairy farms located in Kyunggi Province were examined for the presence of genes encoding staphylicoccal enterotoxins and other exotoxins, 6 types of enterotoxin SEA, SEB, SEC, SEE, TSST, and ETB were detected in the isolates, and 12.5% of them were revealed to possess toxin producing genes as shown in Table 1. A major type of enterotoxin was found to be SEB, SEE and exfoliative toxin B (ETB), none of the 72 isolates carried the genes for SED and ETA. The average distribution of superantigenic exotoxin in the isolates of *S. aureus* was shown to be 38% and ranged from 2% in Denmark to 63% in Norway (Fueyo et al., 2001; Stephan et al., 2001).

Table 2. Detection of genes for enterotoxins among 72 *S. aureus* isolated from An-sung in Korea

Toxin type	Isolates samples(72)		
	positive	size(bp)	ratio(%)
A	1	120	1.4
B	2	478	2.8
C	1	257	1.4
D	0	317	0
E	2	170	2.8
TSST-1	1	350	1.4
ETA	0	119	0
ETB	2	200	2.8
Total	9		12.5
Negative	63		87.5

Isolate CU 1162 and CU 1176 have shown to possess two types of enterotoxins. It revealed in the study of Zshock (2000) that 19% of 94 isolates were single enterotoxin gene harbors and 17% were double enterotoxin gene harbors. It is possible to detect the gene products immunologically by using a variety of enzyme-linked immunosorbent assays and radioimmunoassays (Thompson et al. 1986).But these tests have variable sensitivities for reliability and reproducibility depending on the extent of gene expression.. But toxigenic strains of *S. aureus* with low level of excreted toxins could be easily misidentified by immunologic methods.

The value of genotypic methods in the direct detection of toxin-producing strains of *S. aureus* has not yet been determined but the PCR has an advantage over DNA hybridization in tha t the sensitivity is sufficient to allow the detection of microbial DNA directly in pathological specimens (Schochetmann et al., 1988).This genotypic method allows the detection of gene harboring strains independent of their expression. But a positive results in the PCR is indicative only of the presence of targeted organism and does not indicate its viability or pathogenic toxic potential.

REFERENCES

Ali-Vehmas, T., P. Westphalen, V. myllys and Sandholm. 1997. Binding of *Staphylococcus aureu*s to milk fat globules increases resistance to penicillin-G. J. Dairy Res. 64:253-260.

Alatossava, V., A. Meindl and D. Ring. 2001. Prevalence of *Staphylococcus aureus* and staphylococcal enteritoins in raw pork and uncooked smoked ham-a comparison of classical culturinjg detection and RFLP-PCR. Int. J. Food Microbiol. 68:105-113.

Blanban, N. and A. Rasooly. 2000. Staphylococcal enterotoxins. Int. J. Food Microbiol. 61:1-10.

Betley, M. J. and J. J. Mekalanos. 1988. Nucleotide sequence of the type A staphylococcal enterotoxin gene. J. Bacteriol. 170:34-41.

Coia, J. E., F. Thomson-Carter, D. Baird and D. J. Platt. 1990.

Characterization of Methicillin resistant *Staphylococcus aureus* by biotyping, immunoblotting and restriction enzyme fragmentation patterns. J. Med. Microbiol. 31:125-132.

Fueyo, J. M., M. C. Martin, M. A. Gonzalez-Hevia and M. C. Mendoza. 2001. Enterotoxin production and DNA fingerprinting in *Staphylococcus aureus* isolated from human and food samples. Relations between genetic types and enterotoxins. International J. Food Microbiol. 67:139-145.

Hackbarth, C. J., T. Kpcagoz, S. Kocagoz and H. F. Chambers. 1995. Point mutations in *Staphylococcus* PB2 gene affect penicillin-binding kinetics and are associated with resistance. Antimicrob. Agents Chemother. 39:103-106.

Johnson, W. M., S. D. Tyler, E. P. Ewan, F. E. Ashton, D. R. Pollard and K. R. Rozee. 1991. Detection of genes for enterotoxin, exfoliative toxins, and toxic shock syndrome toxin in Staphylococcus aureus by the Polymerase Chain Reaction. J. Clinical Microbiol. 29(3):426-430.

Junes, C. L. and S. A. Khan. 1986. Nucleotide sequence of enterotoxin B gene from *Staphylococcus aureus*. J. Bacteriol. 166:29-33.

Larsen, H. D., F. M. Aarestrup and N. E. Jensen. 2002. Geographical variation in the presence of genes encoding superantigenic exotoxins and -hemolysin among *Staphylococcus aureus* isolated from bovine mastitis in Europe and USA. Veterinary Microbiology 85:61-67.

Onoue, Y. and M. Mori. 1997. Amino acid requirements for the growth and enterotoxin production by *Staphylococcus aureus* in chemically defined media. Int. J. Food Microbiol. 36: 77-82.

Rasooly, A. and R. S. Rasooly. 1998. Detection and analysis of Staphylococcal enterotoxin A in food by Western immunoblotting. Int. J. Food Microbiol. 41:205-212.

Schochetmann, G., C. Y. Ou and W. K. Jones. 1988. Polymerase chain reaction. J. Infect. Disease 158:1154-1157.

Stephan, R., C. Annemuller, A. A. Hassan and Ch. Lammler. 2001. Characterization of enterotoxigenic *Staphylococcus aureus* strains isolated from bovine mastitis in north-east Switzerland. Veterinary Microbiology 78:373-382

Thompson, N. E., M. Razdan, G. kunstmann, J. M. Aschenbach, M. L. Evenson and M. S. bergdoll. 1986. Detection of stphylococcal enterotoxins by enzyme linked immunosorbent assays and radioimmunoassays; comparison of monoclonal and pllyclonal antibody systems. Appl. Environ. Microbiol. 51:885-890.

Zshock, M., D. Botzler, S. Blocher, J Sommerhauser and H. P. Hamann. 2000. Detection of genes for enterotoxins (ent) and toxic shock syndrome toxin-1 (tst) in mammary isolates of *Staphylococcus aureus* by polymerase-chain-reaction. Int. Dairy J. 10:569-574.