Indian Journal of Fundamental and Applied Life SciencesISSN: 2231– 6345 (Online)
An Open Access, Online International Journal Available at www.cibtech.org/jls.htm
2014 Vol. 4 (3), pp. 683-689/Fardin et al.
Research Article

INVESTIGATING OF ENTEROTOXIN GENES BY MULTIPLEX PCR IN *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM BOVINE MASTITIS

*** Masoud Fardin[1], Mahmood Jamshidian[1], Taghi Zahraei Salehi[1] and Mansour Khakpour[2]**
[1]*Department of Pathobiology, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[2]*Department of Pathobiology, College of Veterinary Medicine, Tabriz Branch, Islamic Azad University, Tabriz, Iran*
**Author for Correspondence*

ABSTRACT
A total of 40 *Staphylococcus aureus* strains isolated from bovine mastitis, were analyzed to investigate the presence of the enterotoxin genes *sea*, *seb*, *seg*, *seh*, *sei* and *sej* using Multiplex PCR. 40% of the isolates were positive for one or more toxin genes. The *seg* gene was found most frequently (32.5%), followed by *sej* (22.5%), *seh* (20%), *seb* (10%), *sei* (10%) and finally, *sea* (7.5%). Our results revealed that Multiplex PCR method is simple, sensitive, low cost, relatively rapid and very specific; in addition, it proved to be able to identify several genes that encode toxin at the same time. The results of this study also showed that *S. aureus* causing mastitis in cows can harbor enterotoxin-encoding genes with *seg* as the most frequent gene observed amongst the investigated isolates. These findings are important for surveillance purposes, since enterotoxin G should be investigated in human staphylococcal food poisoning outbreaks caused by the consumption of cow milk and dairy products.

Keywords: *Staphylococcus Aureus, Enterotoxin Genes, Bovine Mastitis, Multiplex PCR*

INTRODUCTION
Staphylococcus aureus is one of the major bacterial pathogens which cause a variety of infectious in humans and animals. This organism plays an important role in the etiology of infectious bovine mastitis (Atashpaz *et al.*, 2010).
Bovine mastitis is a multifactorial disease, which results in the reduction of milk yield, changes in milk composition and increase in the amount of discarded milk. It imposes serious economic losses for the farmers and the dairy industry (Santos *et al.*, 2008).
Some strains of *Staphylococcus aureus* are pathogenic and responsible for food poisoning by producing enterotoxins (Pereira *et al.*, 2009).
Staphylococcal Enterotoxins (SEs) are a group of single-chain, low-molecular weight proteins (MW, 26900–29600Da), that are very resistant to heat and gastrointestinal proteases such as pepsin, that justifies why they remains active after thermal processing of food and ingestion (El-Huneidi *et al.*, 2006; Fooladi *et al.*, 2010; Pelisser *et al.*, 2009; Rall *et al.*, 2008). SEs are usually divided into the classic (SEA to SEE) and newly described (SEG to SER and SEU) enterotoxins (El-Huneidi *et al.*, 2006).
Several studies have reported the production of SEs or the presence of toxin genes in *Staphylococcus aureus* from milk and derivates associated with mastitis cows in different countries (De Freitas *et al.*, 2008; Karahan *et al.*, 2009; Omoe *et al.*, 2002; Vimercati *et al.*, 2006).
The genes responsible for encoding SEs are often embedded in mobile genetic elements, such as transposons, prophages, plasmids and pathogenicity islands (Fusco *et al.*, 2011), which may be transferred horizontally between staphylococcal strains. So, enterotoxin genes may play an important role in the evolution of *S. aureus* as a pathogen (Zhang *et al.*, 2013).
For the above-mentioned reasons, the evaluation of enterotoxin genes in *S. aureus* isolates can be useful for epidemiological tracing and evolutionary analyses.
There are various methods for the detection of enterotoxigenic bacteria, such as latex agglutination, ELISA, immunodiffusion and RIA. But these methods are not reliable because specific circumstances are necessary for enterotoxin gene expression. However, despite the presence of entrotoxin genes, in specific

circumstances, *S.aureus* may not have the ability to produce toxin which would lead to negative results (Saadati *et al.,* 2011).

In addition, molecular techniques such as PCR and Multiplex PCR are recommended for the detection of *S. aureus* enterotoxin genes (Fooladi *et al.,* 2010). These techniques are rapid, sensitive, specific, and reliable compared to immunological toxin production assays (Pinto *et al.,* 2005). Another advantage of molecular methods is that strains producing low levels of enterotoxin could be identified by these methods (Saadati *et al.,* 2011). Therefore, they constitute very valuable tools for routine applications (Pinto *et al.,* 2005).

In this work, Multiplex PCR method was used to explore the presence of enterotoxin genes *sea*, *seb*, *seg*, *seh*, *sei*, and *sej* in *S. aureus* isolates obtained from milk samples of cows with mastitis in Ardabil, a Northwest provincial city in Iran.

MATERIALS AND METHODS

Bacterial Strains

This study used 40 *S. aureus* strains isolated from milk samples of cows with mastitis that were collected and identified by biochemical tests in a previous study .

Isolates were sub cultured on nutrient agar (NA) and identified by biochemical tests, which included catalase, coagulase, mannitol fermentation, and hemolysin, DNase, lecithinase, protease, and lipase production (Chapaval *et al.,* 2006; El-Huneidi *et al.,* 2006).

DNA Extraction

Bacterial DNA was extracted from overnight broth cultures of the various strains according to the method reported by Atashpaz *et al.,* (2010) with some modification.

The DNA extraction process was carried out as follows:

1) One milliliter overnight culture at 37°C in 5ml brain heart infusion (BHI) broth of each strain was transferred into a centrifuge tube and centrifuged at 3500 rpm for 10 min.

2) The supernatant was discarded and then 800 µl lysing buffer was added to the pellet, mixed thoroughly, the lysing buffer (2% CTAB (Merck, Hohenbrunn, Germany), 100 mM Tris-HCl (Merck, Darmstadt, Germany), 1.4 M NaCl (Merck, Darmstadt, Germany), 1% PVP (AppliChem, Darmstadt, Germany), 20 mM disodium salt of ethylenediaminetetraacetic acid (Na2EDTA; Merck, Darmstadt, Germany), 0.2% LiCl (Merck, Darmstadt, Germany). The pH was adjusted at ~8 for the solution used before autoclaving). Afterwards, the prepared sample was transferred to a 1.5 ml centrifuge tube.

3) The sample was incubated at 65°C for 30 min and gently shacked every 10min.

4) The sample was centrifuged at 10000 rpm for 5 min at 4°C.

5) The supernatant was transferred into a new tube and an equal amount of chloroform– isoamylalchol (Merck, Darmstadt, Germany) (24: 1 vol/vol) was added. Then the tube was gently flipped several times.

6) The sample was centrifuged at 12000 rpm for 8 min at 4°C. The upper phase was then transferred into a new tube.

7) An equal volume of cold (–20°C) isopropanol (Merck, Darmstadt, Germany) was added to precipitate the DNA. Then the sample was stored at –20°C for 30 min.

8) The sample was precipitated at 14000 rpm for 10 min at 4°C.

9) For the first washing step, after the removal of the supernatant, 500 µl of 96% ethanol (Merck, Darmstadt, Germany) (4°C) was added to the sample which was then centrifuged at 12000 rpm for 5 min.

10) For the second washing step, the supernatant was removed and 500 µl of 70% ethanol (4°C) was added to the sample which was then centrifuged at 12000 rpm for 5 min.

11) The supernatant was removed and the pellet was dried at room temperature.

12) The genomic DNA pellet was dissolved in 50 µl TE buffer [10 mM Tris-HCI (pH 8.0), 1 mM EDTA (pH 8.0), (Merck, Darmstadt, Germany)] and DNA solution was stored at –20°C.

Assessing the Quantity and Quality of Extracted DNA

The quantity of the extracted DNA was checked by NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The A260/A280 absorbance ratio was used to determine undesired contaminations.

To evaluate the quality and intactness of the extracted DNA, gel electrophoresis was used. The extracted DNA (5 µl) was loaded on 1% agarose gel (Invitrogen, California, USA), which contained ethidium bromide (1 µg/ml) for DNA staining. And finally, a G:Box™ gel documentation system (Syngene, Cambridge, United Kingdom) was used for image acquisitions.

PCR Primer Design

Six PCR primer sets were used to detect the staphylococcal enterotoxin A gene (*sea*), staphylococcal enterotoxin B gene (*seb*), staphylococcal enterotoxin G gene (*seg*), staphylococcal enterotoxin H gene (*seh*) , staphylococcal enterotoxin I gene (*sei*) and staphylococcal enterotoxin J gene (*sej*) as primers reported previously in the literature.The primers used in this study and their respective amplified products are listed in Table 1.

Table 1: PCR primers sets were used to detect genes *sea, seb, seg, seh, sei* and *sej*

Gene	Primer name	Primer sequences	Size of amplified product	References
sea	SEA-f	GCA GGG AAC AGC TTT AGG C	521 bp	(Monday *et al.*, 1999)
	SEA-r	GTT CTG TAG AAG TAT GAA ACA CG		
seb	SEB-f	ACA TGT AAT TTT GAT ATT CGC ACT G	667 bp	(Løvseth *et al.*, 2004)
	SEB-r	TGC AGG CAT CAT GTC ATA CCA		
seg	SEG-1	AAG TAG ACA TTT TTG GCG TTC C	287 bp	(Omoe *et al.*, 2002)
	SEG-2	AGA ACC ATC AAA CTC GTA TAG C		
seh	SEH-f	CAA CTG CTG ATT TAG CTC AG	360 bp	(Monday *et al.*, 1999)
	SEH-r	GTC GAA TGA GTA ATC TCT AGG		
sei	SEI-1	GGT GAT ATT GGT GTA GGT AAC	454 bp	(Omoe *et al.*, 2002)
	SEI-2	ATC CAT ATT CTT TGC CTT TAC CAG		
sej	SEJ-1	CAT CAG AAC TGT TGT TCC GCT AG	142 bp	(Monday *et al.*, 1999)
	SEJ-2	CTG AAT TTT ACC ATC AAA GGT AC		

Multiplex PCR Amplification

In the recent study, genomic DNA of *S. aureus* strains was amplified in one set of Multiplex PCR. Multiplex PCR reaction was performed in a final volume of 25µl, containing: 1µl of genomic DNA (50 ng/µl), 1 µl of Taq polymerase (5 unit/µl), 0.4 µM of each primers, 200 µM of each dNTP, 2.5 µl of 10X PCR buffer and 1 µl of $MgCl_2$ (1.5 mM).

The amplification program consisted of one initial denaturation at 94°C for 4 min followed by 32 cycles of 50 sec at 94°C for denaturation, 40 sec at 56°C for primer annealing, 180 sec at 72°C for extension and DNA synthesis and final extension at 72°C for 10 min. The products were separated on 1.5% agarose gel containing ethidium bromide (1 µg/ml), then images were taken using a G: Box™ gel documentation system (Syngene, Cambridge, United Kingdom).

RESULTS AND DISCUSSION

Results

In the present study, 40 *S. aureus* strains isolated from milk samples of cows with mastitis were tested for enterotoxin production by Multiplex PCR assay. Testing with specific primers for *sea*, *seb*, *seg*, *seh*, *sei* and *sej* genes were performed by which the existence of a 521bp segment was related to the amplification of a specific fragment of gene *sea* that is responsible for enterotoxin type A (Figure1, lane 9). DNA amplification fragments of 667 bp for staphylococcal enterotoxin are related to B gene (*seb*) (Figure 1, lanes 1,5and 8), 287 bp for staphylococcal enterotoxin G gene (*seg*) (Figure 1, lanes 1 ,5 and 8), 360bp for staphylococcal enterotoxin H gene (*seh*) (Figure 1, lanes 1,4 and 5), 454 bp for staphylococcal enterotoxin I gene (*sei*) (Figure 1, lanes 2 and 8) and 142 bp to staphylococcal enterotoxin J gene (*sej*) (Figure 1, lanes 1,5 and 8). Also, *S. epidermidis* was used as a negative control and did not yield a PCR product (Figure 1, lanes 3 and 7).

A total of 40% of the tested *S. aureus* isolates were positive for one or more toxin genes. 7.5 percent of total isolates were *sea* positive, 10% *seb* positive, 32.5% *seg* positive, 20% *seh* positive, 10% *sei* positive and finally, 22.5% *sej* positive.

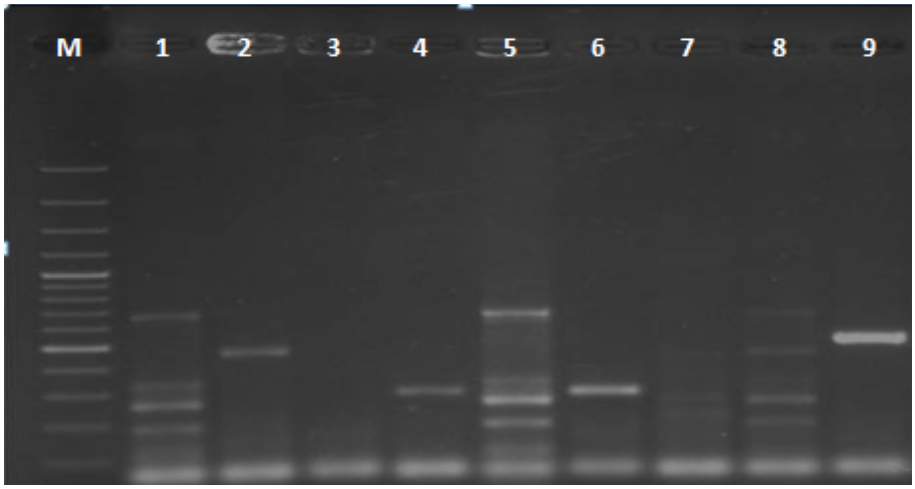

Figure 1: Multiplex PCR assays for the detection of enterotoxin genes in *S.aureus* strains

Lane M, 100 bp DNA marker ; lanes 1 and 5, *sej* (142 bp), *seg* (287 bp), *seh* (360 bp) and *seb* (667 bp); lane 2, *sei* (454 bp); lanes 3 and 7, negative control (*S. epidermidis*); lanes 4 and 6, *seh* (360 bp); lane 8, *sej* (142 bp), *seg* (287 bp), *sei* (454 bp) and *seb* (667 bp); lane 9, *sea* (521 bp).

Discussion

S. aureus is recognized worldwide as a major pathogen causing bovine mastitis, and milk from infected animals is considered to be the main source of enterotoxigenic *S. aureus* of animal origin (Zschock *et al.*, 2005).

In the present study, we have described a Multiplex PCR-based diagnostic protocol to detect the genes for enterotoxins A, B, G, H, I and J in *S. aureus* isolated from milk samples of cows with mastitis. This technique can identify strains harboring the toxin genes and is independent of the expression and secretion of the toxin (Mehrotra *et al.*, 2000).

Multiplex PCR results obtained in this study showed that 40% of *S. aureus* isolates were positive for one or more enterotoxin genes.

This prevalence rate was much lower than 80.2% reported by De Freitas *et al.,* (2008) in the isolates of *Staphylococcus* spp. obtained from cows diagnosed with subclinical mastitis in Brazil.

High frequencies of enterotoxin-encoding genes have also been reported in *S. aureus* isolates of bovine mastitis origin in studies carried out in Japan (71.4%) (Omoe *et al.,* 2002) and Italy (70%) (Vimercati *et al.,* 2006).

But, the percentage obtained in this study was more than what was reported in isolates from bovine mastitis in Turkey (29.3%) (Karahan *et al.,* 2009) and in Brazil (9.4%) (Da Silva *et al.,* 2005).

The predominant enterotoxin type in this study was *seg* (32.5%). This has also been reported as the most frequent gene in *staphylococcus* from cows with subclinical mastitis by De Freitas *et al.,* (2008), who observed *seg* in 35% of strains. The *seg* gene was also reported in 31.4 % of strains by Wang *et al.,* (2009), amongst *Staphylococcus aureus* isolates from milk samples of bovine subclinical mastitis cases. The *sej* gene was detected in 22.5% of *S. aureus* isolates. It was reported in 24.4% of strains isolated from bovine mastitis by Wang *et al.,* (2009) and in 10% of strains by Unal (2013).

The *seh* gene was detected in 20% of *S. aureus* isolates. It was reported in 32% of strains by De Freitas *et al.,* (2008) and in 5% of strains by Unal (2013).

The *sei* gene was detected in 10% of *S. aureus* isolates. This prevalence rate is lower than what was reported by Karahan *et al.,* (2009), who reported the detection of *sei* in 25% of strains, and Wang *et al.,* (2009), who found a prevalence of 31.8%. The *sea* and *seb* genes were detected in 7.5% isolates and 10% isolates, respectively. The *sea* gene, however, was not observed in the *S. aureus* investigated in some studies (Da Silva *et al.,* 2005; Karahan *et al.,* 2009; Omoe *et al.,* 2002). On the other hand, this gene is reported to be the predominant enterotoxin gene amongst *S. aureus* isolates of bovine mastitis origin in other studies (Wang *et al.,* 2009). The variation in reported rate results is probably due to geographical variations. Although the differences in study populations, sampling procedure, the number of samples studied, culture techniques might be effective (Becker *et al.,* 2003; Mehrotra *et al.,* 2000).

Conclusion

Multiplex PCR method was used to investigate the presence of *sea*, *seb*, *seg*, *seh*, *sei* and *sej* genes in *S. aureus* isolated from milk samples of cows with mastitis for the first time in Iran. Our results revealed that this method is simple, sensitive, low cost, relatively rapid and very specific and that it can identify several genes that encode toxin at the same time. Besides, on the basis of our examinations and available scientific literature, the conclusion reached was that *S. aureus* causing mastitis in cows can harbor enterotoxin-encoding genes with *seg* as the most frequent gene observed amongst the investigated isolates. These finding are important for surveillance purposes, since enterotoxin G should be investigated in human staphylococcal food poisoning outbreaks caused by the consumption of cow milk and dairy products.

REFERENCES

Atashpaz S, Khani S, Barzegari A, Barar J, Vahed SZ, Azarbaijani R and Omidi Y (2010). A robust universal method for extraction of genomic DNA from bacterial species. *Microbiology* **79**(4) 538-542.

Becker K, Friedrich AW, Lubritz G, Weilert M, Peters G and Von Eiff C (2003). Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. *Journal of Clinical Microbiology* **41**(4) 1434-1439.

Chapaval L, Moon DH, Gomes JE, Duarte FR and Tsai SM (2006). Use of PCR to detect classical Enterotoxins Genes (ENT) and Toxic Shock Syndrome Toxin-1 Gene (TST-1) in *Staphylococcus aureus* Isolated from crude milk and determination of toxin productivities of *S. aureus* Isolates harboring these genes. *Arquivos do Instituto Biologico, Sao Paulo* **73**(2) 165-169.

Da Silva ER, Carmo LSD and Da Silva N (2005). Detection of the enterotoxins A, B, and C genes in *Staphylococcus aureus* from goat and bovine mastitis in Brazilian dairy herds. *Veterinary Microbiology* **106**(1) 103-107.

De Freitas MF, Luz IDS, Silveira-Filho VDM, Júnior J WP, Stamford TL, Mota RA and Leal-Balbino TC (2008). Staphylococcal toxin genes in strains isolated from cows with subclinical mastitis. *Pesquisa Veterinária Brasileira* **28**(12) 617-621.

El-Huneidi W, Bdour S and Mahasneh A (2006). Detection of enterotoxin genes *seg seh sei* and *sej* and of a novel aroA genotype in Jordanian clinical isolates of *Staphylococcus aureus*. *Diagnostic Microbiology and Infectious Disease* **56**(2) 127-132.

Fooladi AI, Tavakoli HR and Naderi A (2010). Detection of enterotoxigenic *Staphylococcus aureus* isolates in domestic dairy products. *Iranian Journal of Microbiology* **2**(3) 137.

Fusco V, Quero GM, Morea M, Blaiotta G and Visconti A (2011). Rapid and reliable identification of *Staphylococcus aureus* harbouring the enterotoxin gene cluster (egc) and quantitative detection in raw milk by real time PCR. *International Journal of Food Microbiology* **144**(3) 528-537.

Karahan M, Açık MN and Cetinkaya B (2009). Investigation of toxin genes by polymerase chain reaction in *Staphylococcus aureus* strains isolated from bovine mastitis in Turkey. *Foodborne Pathogens and Disease* **6**(8) 1029-1035.

Løvseth A, Loncarevic S and Berdal KG (2004). Modified multiplex PCR method for detection of pyrogenic exotoxin genes in staphylococcal isolates. *Journal of Clinical Microbiology* **42**(8) 3869-3872.

Mehrotra M, Wang G and Johnson WM (2000). Multiplex PCR for detection of genes for *staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. *Journal of Clinical Microbiology* **38**(3) 1032-1035.

Monday SR and Bohach GA (1999). Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. *Journal of Clinical Microbiology* **37**(10) 3411-3414.

Omoe K, Ishikama M, Shimoda Y, Hu DL, Ueda S and Shinagawa K (2002). Detection of *seg*, *seh* and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolate harboring *seg*, *seh* or *sei* genes. *Journal of Clinical Microbiology* **40**(3) 857–862.

Pelisser MR, Klein CS, Ascoli KR, Zotti TR and Arisi ACM (2009). Ocurrence of *Staphylococcus aureus* and multiplex PCR detection of classic enterotoxin genes in cheese and meat products. *Brazilian Journal of Microbiology* **40**(1) 145-148.

Pereira V, Lopes C, Castro A, Silva J, Gibbs P and Teixeira P (2009). Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of *Staphylococcus aureus* isolates from various foods in Portugal. *Food Microbiology* **26**(3) 278-282.

Pinto B, Chenoll E and Aznar R (2005). Identification and typing of food-borne *Staphylococcus aureus* by PCR-based techniques. *Systematic and Applied Microbiology* **28**(4) 340-352.

Rall VLM, Vieira FP, Rall R, Vieitis RL, Fernandes Jr A, Candeias JMG and Araújo Jr JP (2008). PCR detection of staphylococcal enterotoxin genes in *Staphylococcus aureus* strains isolated from raw and pasteurized milk. *Veterinary Microbiology* **132**(3) 408-413.

Saadati M, Barati B, Doroudian M, Shirzad H, Hashemi M, Hosseini SM and Imani S (2011). Detection of *Sea, Seb, Sec, Seq* genes in *staphylococcus aureus* isolated from nasal carriers in Tehran province, Iran; by multiplex PCR. *Journal of Paramedical Sciences* **2**(2).

Santos OCDS, Barros EM, Brito MAVP, Bastos MDCDF, Dos Santos KRN and Giambiagi-deMarval M (2008). Identification of coagulase-negative staphylococci from bovine mastitis using RFLP-PCR of the *groEL* gene. *Veterinary Microbiology* **130**(1) 134-140.

Unal N (2013). Investigation of some toxins genes and methicillin resistance gene in *Staphylococcus aureus* isolates from cows with subclinical mastitis. *Ankara Universite Veteriner Fakultesi Dergisi* **60**(1) 21-26.

Vimercati C, Cremonesi P, Castiglioni B, Pisoni G, Boettcher PJ, Stella A and Moroni P (2006). Molecular typing of *Staphylococcus aureus* isolated from cows, goats and sheep with intramammary infections on the basis of gene polymorphisms and toxins genes. *Journal of Veterinary Medicine, Series B* **53**(9) 423-428.

Wang SC, Wu CM, Xia SC, Qi YH, Xia LN and Shen JZ (2009). Distribution of superantigenic toxin genes in *Staphylococcus aureus* isolates from milk samples of bovine subclinical mastitis cases in two major diary production regions of China. *Veterinary Microbiology* **137**(3) 276-281.

Zhang Y, Cheng S, Ding G, Zhu M, Pan X and Zhang L (2013). Molecular analysis and antibiotic resistance investigation of *Staphylococcus aureus* isolates associated with staphylococcal food poisoning and nosocomial infections. *African Journal of Biotechnology* **10**(15) 2965-2972.

Zschock M, Kloppert B, Wolter W, Hamann HP and Lammler C (2005). Pattern of enterotoxin genes *seg*, *seh*, *sei* and *sej* positive *Staphylococcus aureus* isolated from bovine mastitis. *Veterinary Microbiology* **108** 243–249.