Development of a routine laboratory direct detection system of staphylococcal enterotoxin genes

Akifumi Nakayama,^{1†} Akiko Okayama,¹ Misao Hashida,¹
Yasuzumi Yamamoto,¹ Hisakatsu Takebe,¹ Takashi Ohnaka,²
Tomoyuki Tanaka² and Shunsuke Imai¹

Correspondence

Akifumi Nakayama
pyonchan@asint.jp

¹Nara Prefectural Institute for Hygiene and Environment, 57-6 Ohmori-cho, Nara City, Nara 630-8131, Japan

²Sakai City Institute of Public Health, 3-2-8 Kaichyo Higashi, Sakai City, Osaka 590-0953, Japan

A novel direct detection system has been developed for eight staphylococcal enterotoxin (SE)-encoding genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh* and *sei*) in milk. Specific detection by real-time PCR was successful for all SE-encoding genes in the reference strains. Furthermore, a novel DNA-preparation method with good reproducibility [coefficients of variation 0.31, 0.99 and 1.21 % at 10^6 , 10^4 and 10^2 c.f.u. (ml milk sample)⁻¹, respectively] was developed to overcome PCR inhibition in the milk samples. The combination of this DNA-preparation method and real-time PCR resulted in high sensitivity [between 1.1×10^2 and 1.0×10^4 c.f.u. (ml milk sample)⁻¹] and allowed the completion of the entire procedure within 4 h. Results of an evaluation of this method for the detection of SE-encoding genes using known outbreak milk samples produced results showing good correspondence with the reversed passive latex agglutination assay. In addition, this newly developed system can be applied to clinical samples such as faeces and vomit. Consequently, the system should be useful in the routine direct detection of SE-encoding genes in food-borne-poisoning samples.

Received 28 January 2005

Accepted 21 October 2005

INTRODUCTION

Food-borne poisoning by staphylococcal enterotoxins (SEs) is an important hazard. SEs, the emetic toxins produced by *Staphylococcus aureus*, have been divided into five serological types (SEA, SEB, SEC, SED and SEE) (Bergdoll, 1983). In recent years, new types of SEs (from SEG to SEIU) have been reported (Jarraud *et al.*, 2001; Munson *et al.*, 1998; Orwin *et al.*, 2001; Ren *et al.*, 1994; Su & Wong, 1995; Zhang *et al.*, 1998; Sergeev *et al.*, 2004; Omoe *et al.*, 2003; Lina *et al.*, 2004), but the emetic activity of several of these toxins has not been confirmed. However, various studies have shown that the newly described *seg* and *sei* genes are commonly found in food-borne and clinical isolates of *S. aureus* (Jarraud *et al.*, 2001; McLauchlin *et al.*, 2000; Omoe *et al.*, 2002; Rosec & Giraud, 2002; Becker *et al.*, 2003), suggesting that they have roles as pathogenic factors in staphylococcal food poisoning. Thus, the establishment of a method to detect eight enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*

and *sei*) is vital for the analysis of staphylococcal food poisoning. Recently, Letertre *et al.* (2003a) reported the detection of SE-encoding genes based on 5' nuclease multiplex PCR. However, this method cannot detect SE-encoding genes directly from samples because it requires a culturing process. Direct detection of pathogenic genes by PCR is hampered by inhibitors contained in the samples (Rossen *et al.*, 1992; Wilson, 1997). Among the food samples, milk contains high concentrations of proteins and fats, which are major inhibitors of the PCR assay. In 2000, a large-scale and widespread outbreak of staphylococcal food poisoning caused by the ingestion of dairy products, including low-fat milk and yogurt beverages, occurred in Japan (Asao *et al.*, 2003) affecting a total of 13 420 persons. This incident eloquently indicated the need for a rapid and direct detection method for routine laboratory analysis.

The aim of this study was to establish a system to directly detect eight SE-encoding genes in the food-borne-poisoning samples. To reach this objective, a novel DNA-preparation method and an real-time PCR method were developed, and these were evaluated using milk samples from a known outbreak (Asao *et al.*, 2003) and clinical samples.

[†]Present address: Clinical Laboratory, Nara Prefectural Hospital, 1-30-1 Hiramatsu, Nara City, Nara 631-0846, Japan.

Abbreviations: Ct, threshold cycle; MGB, minor groove binder; RPLA, reversed passive latex agglutination; SE, staphylococcal enterotoxin.

METHODS

Bacterial strains. Five *S. aureus* strains (FRI-100, ATCC 14458, FRI-137, ATCC 23235 and FRI-326) were kindly provided by the Tokyo Metropolitan Institute of Public Health, Tokyo, Japan and were used as positive controls. The *S. aureus* FRI-137 strain has been reported to possess the following four enterotoxin genes (Letertre *et al.*, 2003b): *sec*, *seg*, *seh* and *sei*. The following strains were used for negative controls: the staphylococci *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus capitis* (ATCC 35661) and *Staphylococcus xylosum* (ATCC 29971), and five common food-borne pathogens, *Bacillus cereus* (ATCC 11778), *Campylobacter jejuni* (ATCC 33291), *Clostridium perfringens* (ATCC 13124), *Vibrio parahaemolyticus* (ATCC 17802) and *Yersinia enterocolitica* (ATCC 9610).

Milk samples. Pasteurized milk was purchased from the supermarket and stored at 4 °C until needed for the preparation of milk samples with known concentrations (10^6 c.f.u. ml⁻¹) of *S. aureus* cells for the construction of standard curves. In addition, milk samples that gave rise to the staphylococcal food-poisoning outbreak (in the Kansai district of Japan from the end of June to the beginning of July 2000) described in Asao *et al.* (2003) were used for the evaluation of the direct detection system for SE-encoding genes. These milk samples were stored at -80 °C until needed.

Construction of standard curves. *S. aureus* cells (positive control strains) cultured in tryptone soya broth at 37 °C were harvested at the exponential growth phase by centrifugation and resuspended in pasteurized milk. After DNA extraction from the milk samples, six serial dilutions of each DNA solution [10^6 , 10^5 , 10^4 , 10^3 , 10^2 or 10^1 c.f.u. (ml milk)⁻¹] were made and analysed in triplicate for the production of a standard curve.

Identification of *S. aureus* strains from clinical samples. *S. aureus* strains from clinical samples were checked by the Gram-staining test and the catalase production test (Palk, 1980). These strains were identified by coagulase production (Staphylase test; Oxoid), and by characterization using the VITEK system I (bioMérieux).

Detection of SEs by reversed passive latex agglutination (RPLA). SEs, SEA, SEB, SEC, SED and SEE, were detected using the Enterotox-F RPLA kit (Denka Seiken), following the manufacturer's instructions, in the staphylococcal strains *S. aureus*, *S. epidermidis*, *S. capitis* and *S. xylosum*. SEs in milk samples were detected using a modified RPLA method (Asao *et al.*, 2003).

Oligonucleotide primers and fluorescence-labelled probes. Specific primer pairs, having identical annealing temperatures and working simultaneously under a single thermal-cycling condition, were designed using Primer Express software, version 2.0 (Applied Biosystems), based on the published sequences of SE-encoding genes (Betley & Mekalanos, 1988; Ranelli *et al.*, 1985; Bohach & Schlievert, 1987; Bayles & Iandolo, 1989; Couch *et al.*, 1988; Omoe *et al.*, 2002). Fluorescence (fluorescein phosphoramidites)-labelled probes were designed with minor groove binder (MGB) to increase the specificity of probe hybridization and to enhance spectral performance. Specific MGB probes were synthesized by Applied Biosystems. The annealing temperatures of these fluorescence-labelled probes were approximately 6–11 °C higher than those of the PCR primers, allowing the probes to hybridize with the target region of the amplicon during the annealing/extension stage of each PCR cycle. The PCR primers and fluorescence-labelled probes used in this study are listed in Table 1.

Real-time PCR conditions. Reaction mixtures (50 µl) contained 5 µl DNA template, 1 × TaqMan buffer (Mg²⁺ free), 5 mM MgCl₂, 200 µM dATP, dCTP and dGTP, 400 µM dUTP, 400 nM each specific primer pair, 200 nM of the appropriate TaqMan MGB probe, 0.5 U uracil-N-glycosylase, and 0.75 U AmpliTaq Gold DNA polymerase

(Applied Biosystems). Separate reactions were carried out for each of the eight genes to retain amplification efficiency. Cycling conditions were: 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Reactions were carried out in a 96-well MicroAmp optical plate (Applied Biosystems) using the ABI Prism 7000 sequence detector (Applied Biosystems).

DNA-preparation method. Through this study, we attempted to establish a direct detection system for SE-encoding genes in samples collected from cases of staphylococcal food poisoning. To achieve this goal, a new DNA-preparation method was developed. This consisted of the following three basic steps: step 1, alkaline treatment of samples to achieve cell lysis and inhibit nuclease activity; step 2, petroleum ether extraction to eliminate fats and proteins in the sample; step 3, application of a commercial kit for DNA purification and concentration. In this procedure, each milk sample (100 µl) was added to an equal volume 0.2 M sodium hydroxide, and the solution was incubated at 37 °C for 20 min. The alkaline treated sample (200 µl) was neutralized with 10 µl 3 M sodium acetate (pH 5.4), extracted with 1.0 ml petroleum ether, and then centrifuged at 13 000 g for 10 min at 25 °C. The aqueous phase was transferred to a fresh tube. Bacterial DNA was purified from the aqueous solution using a Wizard SV genomic DNA purification kit (Promega). Finally, DNA was eluted in 50 µl sterile distilled water and stored at -20 °C.

Cloning and sequencing of PCR products. Real-time PCR products from reference strains were cloned into the pCRII-TOPO plasmid vector (Invitrogen) according to the manufacturer's instructions. DNA sequencing of the cloned amplification products was carried out by the Dragon Genomics Center (Takara Bio).

RESULTS AND DISCUSSION

Specificity of real-time PCR

The specificity of real-time PCR was tested for the positive and negative control strains. The eight SE-encoding genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh* and *sei*) were detected in the positive control strains and not in the negative control strains (Table 2). In addition, the DNA sequences of all real-time PCR products showed complete agreement with the sequences of the corresponding region of each SE-encoding gene (data not shown). Therefore, the eight pairs of primers designed in this study were determined to be completely specific for each SE-encoding gene.

Sensitivity and reproducibility of the direct detection system

Standard curves of threshold cycle (Ct) values versus the log₁₀ of the c.f.u. values were plotted for milk samples inoculated with 10^6 c.f.u. *S. aureus* (ml milk)⁻¹ for evaluation of the sensitivity and reproducibility of the combined newly developed DNA-preparation method and real-time PCR. All SE-encoding gene standard curves based on 10-fold dilutions of DNA showed a linear relationship between log₁₀ c.f.u. and Ct value. The detection limit for each SE-encoding gene was between 1.1×10^2 (Ct = 39.44) and 1.0×10^4 (Ct = 37.92) c.f.u. (ml milk sample)⁻¹ (Table 3), and the R² values of the linear regressions were greater than 0.9736. Furthermore, the reproducibility of *S. aureus* (for strain ATCC 14458; n = 5) cellular DNA-preparation from milk at

Table 1. Real-time PCR primers and fluorescence-labelled probes specific for the SE-encoding genes

Genotype	Primer/probe	Oligonucleotide sequence (5'-3')*	T _m value	Position†	GenBank accession no.
<i>sea</i>	eta-F	TTTGGAACCGTTAAAACGAATAAG	59	489–513	M18970
	eta-R	TTTCCTGTAAATAACGTCTTGCTTGA	59	543–568	
	eta-T	FAM-CTGTTCAGGAGTTGGATC-MGB	69	524–541	
<i>seb</i>	etb-F	AGGTGACTGCTCAAGAATTAGATTACC	58	785–811	M11118
	etb-R	AAGGCGAGTTGTTAAATTCATAGAGTT	58	842–868	
	etb-T	FAM-AACTCGTCACTATTTGGTG-MGB	69	813–831	
<i>sec</i>	etc-F	GGCGATAAGTTTGACCAATCTAAATAT	58	811–837	X05815
	etc-R	AAGGTGGACTTCTATCTTCACACTTTT	58	864–900	
	etc-T	FAM-TGTACAACGACAATAAA-MGB	68	845–861	
<i>sed</i>	etd-F	CACAAGCAAGGCGCTATTTG	58	836–855	M28521
	etd-R	TCGGGAAAATCACCTTAACA	58	966–986	
	etd-T	FAM-ATACAGCGCGGAAA-MGB	64	901–914	
<i>see</i>	ete-F	CTTTGGCGGTAAGGTGCAA	58	594–612	M21319
	ete-R	ACCGTGGACCCCTTCAGAAGA	58	634–653	
	ete-T	FAM-AGGCTTGATTGTGTTTCA-MGB	69	615–632	
<i>seg</i>	etg-F	CAATCGACAATAGACAATCACTTGG	59	392–416	AB060535
	etg-R	GCAGAACCATCAAACCTCGTATAGCT	59	500–524	
	etg-T	FAM-TGGCTCACTAAAGAAA-MGB	68	481–496	
<i>seh</i>	eth-F	TAAATAGTGAAAAATTGGCACAGGAA	59	296–321	AB060536
	eth-R	AATTCTTGCAATGTCACATTTTCTT	58	388–413	
	eth-T	FAM-CTAATGTTTGGGTAGATGGT-MGB	69	335–354	
<i>sei</i>	eti-F	GGGCCACTTATCAGGACAATACT	59	242–265	AB060537
	eti-R	GTTTTATGTTTGCCATTAAACCAAA	59	296–320	
	eti-T	FAM-AAATTCTGCTAGAAAAATCCCTAT-MGB	69	267–290	

*The fluorogenic probes (T; in bold) were 5' end-labelled with 6-carboxyfluorescein (fluorescein phosphoramidites; FAM) and 3' end-labelled with MGB.

†Positions correspond to the nucleotide numbers downstream from the ATG start codon of the respective enterotoxin genes.

Table 2. Specificity of real-time PCR for the identification of SE-encoding genes

Strain	RPLA	Real-time PCR
<i>S. aureus</i>		
FRI-100	SEA	<i>sea</i>
ATCC 14458	SEB	<i>seb</i>
FRI-137	SEC	<i>sec, seg, seh, sei</i>
ATCC 23235	SED	<i>sed, seg, sei</i>
FRI-326	SEE	<i>see</i>
Other staphylococci		
<i>S. epidermidis</i>	—	—
<i>S. capitis</i>	—	—
<i>S. xylosus</i>	—	—
Common food-borne pathogens		
<i>B. cereus</i>	ND	—
<i>Campylobacter jejuni</i>	ND	—
<i>Clostridium perfringens</i>	ND	—
<i>V. parahaemolyticus</i>	ND	—
<i>Y. enterocolitica</i>	ND	—

ND, Not determined; —, negative result.

levels of 10^6 , 10^4 and 10^2 c.f.u. (ml milk)⁻¹ had coefficients of variation of 0.31, 0.99 and 1.21 %, respectively. These results, good sensitivity and a high level of reproducibility, confirmed that this system is suitable for the direct detection of SE-encoding genes.

Direct detection of SE-encoding genes in milk samples

Since our system was targeted toward food poisoning samples, milk samples from a known staphylococcal outbreak were tested to demonstrate its applicability. Using previously available methods, SEA was detected in staphylococcal-outbreak milk samples by the modified RPLA method (Asao *et al.*, 2003). For the modified RPLA method, a rotary evaporator was used to concentrate and extract the SE in the samples, and this method identified SEA as the only serotype detected in 20-fold concentrated milk samples. However, *S. aureus* strains were not isolated by the culture method. Thus, the detection of SE-encoding genes in the milk samples using our system was compared with RPLA results. Ten milk samples were examined for the presence of SE-encoding genes and SE proteins. As shown in Table 4, all milk samples producing positive results in RPLA reactions also showed

Table 3. Detection limits and standard curve parameters of the SE-encoding genes in milk

SE-encoding genes	Detection limit (c.f.u. ml ⁻¹)	Standard curve*		
		Slope	Intercept	R ²
<i>sea</i>	6.3 × 10 ²	-2.94	48.91	0.9848
<i>seb</i>	1.1 × 10 ²	-3.02	48.55	0.9831
<i>sec</i>	1.7 × 10 ³	-3.10	54.46	0.9774
<i>sed</i>	1.0 × 10 ⁴	-3.02	52.44	0.9894
<i>see</i>	2.8 × 10 ²	-3.50	53.28	0.9872
<i>seg</i>	1.5 × 10 ³	-3.24	53.91	0.9824
<i>seh</i>	2.5 × 10 ³	-3.13	51.24	0.9736
<i>sei</i>	1.1 × 10 ³	-3.32	55.13	0.9792

*Standard curve (Ct value versus log₁₀ c.f.u. number) constructed for each SE-encoding gene.

positive real-time PCR results, while two milk samples (Sakai-11 and -29) were negative for both assays. These milk samples with negative results contained *S. aureus* at a considerably lower level or did not contain it at all. In addition, the real-time PCR results indicated that the positive milk samples contained not only *sea*, but also *seg*, *seh* and *sei* genes.

Application to clinical samples

We applied a newly developed system for the detection of SE-encoding genes to clinical samples. A total of twenty clinical samples (19 diarrhoea faeces and 1 vomit sample), from which *S. aureus* had been isolated, were used. Each clinical sample was suspended with an equal volume of PBS, pH 7.0, and then subjected to our direct PCR system. *S. aureus* strains (5 colonies) isolated from each clinical sample were also examined for the existence of SE-encoding genes by real-time PCR. The real-time PCR results of the direct detection showed good correspondence with results of previously isolated *S. aureus* strains from 20 clinical samples

Table 4. Comparison of the results from RPLA and real-time PCR for the known outbreak milk samples

Sample	RPLA*	Real-time PCR†
Sakai-3	SEA	<i>sea, seg, seh, sei</i>
Sakai-4	SEA	<i>sea, seg, seh, sei</i>
Sakai-5	SEA	<i>sea, seg, seh, sei</i>
Sakai-6	SEA	<i>sea, seg, seh, sei</i>
Sakai-7	SEA	<i>sea, seg, seh, sei</i>
Sakai-10	SEA	<i>sea, seg, seh, sei</i>
Sakai-11	—	—
Sakai-23	SEA	<i>sea, seg, seh, sei</i>
Sakai-29	—	—
Sakai-33	SEA	<i>sea, seg, seh, sei</i>

—, Negative result.

*SEs were detected using the method of Asao *et al.* (2003).

†SE-encoding genes were detected by the system developed in this study.

(Table 5). SE-encoding genes were detected, using the direct PCR system, in all 11 positive samples, and in none of the 9 negative samples. Furthermore, the direct PCR system also identified more SE-encoding genes than did the isolation method for three of the samples, indicating the possibility that other enterotoxigenic *S. aureus* strains without viability were present in the clinical samples.

In conclusion, we have described the development of a system for the direct detection of SE-encoding genes in milk

Table 5. Comparison of SE-encoding genes detected in clinical samples using previously isolated strains and using the direct detection method

Sample	SE-encoding gene detected	
	Direct method	Isolated strain
Faeces	—	—
Faeces	<i>seb, sec, seg, sei</i>	<i>seb, seg, sei</i>
Faeces	<i>sea, seb, seg, sei</i>	<i>sea, seg, sei</i>
Faeces	<i>sea, seb, seg, sei</i>	<i>sea, seg, sei</i>
Faeces	—	—
Faeces	<i>sea</i>	<i>sea</i>
Faeces	<i>seb</i>	<i>seb</i>
Faeces	<i>sea</i>	<i>sea</i>
Faeces	<i>seb</i>	<i>seb</i>
Vomit	<i>sec, seg, sei</i>	<i>sec, seg, sei</i>
Faeces	—	—
Faeces	—	—
Faeces	—	—
Faeces	—	—
Faeces	<i>sea</i>	<i>sea</i>
Faeces	<i>seb</i>	<i>seb</i>
Faeces	<i>seg, sei</i>	<i>seg, sei</i>
Faeces	—	—
Faeces	—	—
Faeces	—	—

—, Negative result.

samples. The system is composed of a novel DNA-preparation method and real-time PCR, and showed good specificity and sensitivity for the detection of SE-encoding genes. In addition, the DNA-preparation method had a high level of reproducibility based on the determined coefficients of variation. Furthermore, this method was also applicable to clinical samples with high-levels of PCR inhibitors. Ramesh *et al.* (2002) reported a method of DNA extraction from milk for the detection of *S. aureus* and *Y. enterocolitica* by PCR. Their method successfully overcame the inhibition on the PCR assay. Because it detects a staphylococcal nuclease gene as a target of PCR, the sensitivity of the PCR is higher than in our method. However, the DNA-preparation step of their method is time consuming. Our newly developed direct detection system allows the completion of the entire procedure (DNA preparation and real-time PCR) within 4 h. Thus, our system is convenient for routine laboratory applications, especially in the investigation of food poisoning outbreaks caused by *S. aureus*.

ACKNOWLEDGEMENTS

We thank Dr Keizo Yamamoto (Department of Chemistry, Nara Medical University, Nara, Japan) for valuable suggestions in this study.

REFERENCES

- Asao, T., Kumeda, Y., Kawai, T., Shibata, T., Oda, H., Haruki, K., Nakazawa, H. & Kozaki, S. (2003). An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. *Epidemiol Infect* **130**, 33–40.
- Bayles, K. W. & Iandolo, J. J. (1989). Genetic and molecular analyses of the gene encoding staphylococcal enterotoxin D. *J Bacteriol* **171**, 4799–4806.
- Becker, K., Friedrich, A. W., Lubritz, G., Weilert, M., Peters, G. & von Eiff, C. (2003). Prevalence of genes coding for pyrogenic toxin superantigens and exfoliative toxins in blood and nasal strains of *Staphylococcus aureus*. *J Clin Microbiol* **41**, 1434–1439.
- Bergdoll, M. S. (1983). Enterotoxins. In *Staphylococci and Staphylococcal Infections*, pp. 559–598. Edited by C. S. F. Easton & C. Adlam. London: Academic Press.
- Betley, M. J. & Mekalanos, J. J. (1988). Nucleotide sequence of the type A staphylococcal enterotoxin gene. *J Bacteriol* **170**, 34–41.
- Bohach, G. A. & Schlievert, P. M. (1987). Nucleotide sequence of the staphylococcal enterotoxin C1 gene and relatedness to other pyrogenic toxins. *Mol Gen Genet* **209**, 15–20.
- Couch, J. L., Soltis, M. T. & Betley, M. J. (1988). Cloning and nucleotide sequence of the type E staphylococcal enterotoxin gene. *J Bacteriol* **170**, 2954–2960.
- Jarraud, S., Peyrat, M. A., Lim, A. & 7 other authors (2001). *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J Immunol* **166**, 669–677.
- Letertre, C., Perelle, S., Dilasser, F. & Fach, P. (2003a). A strategy based on 5' nuclease multiplex PCR to detect enterotoxin genes *sea* to *sej* of *Staphylococcus aureus*. *Mol Cell Probes* **17**, 227–235.
- Letertre, C., Perelle, S., Dilasser, F. & Fach, P. (2003b). Detection and genotyping by real-time PCR of the staphylococcal enterotoxin genes *sea* to *sej*. *Mol Cell Probes* **17**, 139–147.
- Lina, G., Bohach, G. A., Nair, S. P., Hiramatsu, K., Jouvin-Marche, E. & Mariuzza, R. (2004). Standard nomenclature for the superantigens expressed by staphylococcus. *J Infect Dis* **189**, 2334–2336.
- McLauchlin, J., Narayanan, G. L., Mithani, V. & O'Neill, G. (2000). The detection of enterotoxins and toxic shock syndrome toxin genes in *Staphylococcus aureus* by polymerase chain reaction. *J Food Prot* **63**, 479–488.
- Munson, S. H., Tremaine, M. T., Betley, M. J. & Welch, R. A. (1998). Identification and characterization of staphylococcal enterotoxin type G and I from *Staphylococcus aureus*. *Infect Immun* **66**, 3337–3348.
- Omoe, K., Ishikawa, M., Shimoda, Y., Hu, D.-L., Ueda, S. & Shinagawa, K. (2002). Detection of *seg*, *seh*, and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolates harboring *seg*, *seh*, or *sei* genes. *J Clin Microbiol* **40**, 857–862.
- Omoe, K., Hu, D. L., Takahashi-Omoe, H., Nakane, A. & Shinagawa, K. (2003). Identification and characterization of a new staphylococcal enterotoxin-related putative toxin encoded by two kinds of plasmids. *Infect Immun* **71**, 6088–6094.
- Orwin, P. M., Leung, D. Y. M., Donahue, H. L., Novick, R. P. & Schlievert, P. M. (2001). Biochemical and biological properties of staphylococcal enterotoxin K. *Infect Immun* **69**, 360–366.
- Palk, G. (1980). Reagents, strains and miscellaneous test procedures. In *Manual of Clinical Microbiology*, 3rd edn, pp. 1000–1024. Edited by E. Lennette, A. Balows, W. Hausler Jr & J. Truant. Washington, DC: American Society for Microbiology.
- Ramesh, A., Padmapriya, B. P., Chandrashekar, A. & Varadaraj, M. C. (2002). Application of a convenient DNA extraction method and multiplex PCR for the direct detection of *Staphylococcus aureus* and *Yersinia enterocolitica* in milk samples. *Mol Cell Probes* **16**, 307–314.
- Ranelli, D. M., Jones, C. L., Johns, M. B., Mussey, G. J. & Khan, S. A. (1985). Molecular cloning of staphylococcal enterotoxin B gene in *Escherichia coli* and *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **82**, 5850–5854.
- Ren, K., Bannan, J. D., Pancholi, V., Cheung, A. L., Robbins, J. C., Fischetti, V. A. & Zabriskie, J. B. (1994). Characterization and biological properties of a new staphylococcal enterotoxin. *J Exp Med* **180**, 1675–1683.
- Rosec, J. P. & Giraud, O. (2002). Staphylococcal enterotoxin genes of classical and new types detected by PCR in France. *Int J Food Microbiol* **77**, 61–70.
- Rossen, L., Norskov, P., Holmstrom, K. & Rasmussen, O. F. (1992). Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *Int J Food Microbiol* **17**, 37–45.
- Sergeev, N., Volokhov, D., Chizhikov, V. & Rasooly, A. (2004). Simultaneous analysis of multiple staphylococcal enterotoxin genes by an oligonucleotide microarray assay. *J Clin Microbiol* **42**, 2134–2143.
- Su, Y. C. & Wong, A. C. L. (1995). Identification and purification of a new staphylococcal enterotoxin, H. *Appl Environ Microbiol* **61**, 1438–1443.
- Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol* **63**, 3741–3751.
- Zhang, S., Iandolo, J. J. & Stewart, G. C. (1998). The enterotoxin D plasmid of *Staphylococcus aureus* encodes a second enterotoxin determinant (*sej*). *FEMS Microbiol Lett* **168**, 227–233.