APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 2006, p. 6593–6599 Vol. 72, No. 10
0099-2240/06/$08.00+0 doi:10.1128/AEM.03068-05

Immunoquantitative Real-Time PCR for Detection and Quantification of *Staphylococcus aureus* Enterotoxin B in Foods

Andreja Rajkovic,[1] Benaissa El Moualij,[2] Mieke Uyttendaele,[1]* Philippe Brolet,[2] Willy Zorzi,[2] Ernst Heinen,[2] Ellen Foubert,[1] and Johan Debevere[1]

Laboratory of Food Microbiology and Food Preservation, Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University, Ghent,[1] and Laboratory of Human Histology, University of Liege, Liege,[2] Belgium

Received 29 December 2005/Accepted 31 July 2006

A real-time immunoquantitative PCR (iqPCR) method for detection of *Staphylococcus aureus* enterotoxin B (SEB) was developed and evaluated using both pure cultures and foods. The assay consisted of immunocapture of SEB and real-time PCR amplification of the DNA probe linked to the detection antibody. iqPCR was compared to an in-house enzyme-linked immunosorbent assay (ELISA) using the same couple of capture-detection antibodies and to commercial kits for detection of *S. aureus* enterotoxins (SE). The iqPCR was approximately 1,000 times more sensitive (<10 pg ml^{-1}) than the in-house ELISA and had a dynamic range of approximately 10 pg ml^{-1} to approximately 30,000 pg ml^{-1}. iqPCR was not inhibited by any of the foods tested and was able to detect SEB present in these foods. No cross-reactivity with SE other than SEB was observed. Application of iqPCR for detection of SEB in cultures of *S. aureus* revealed the onset of SEB production after 4 h of incubation at 22, 37, and 42°C, which was in the first half of the exponential growth phase. The total amounts of SEB produced by the two strains tested were larger at 42°C than at 37°C and were strain dependent.

The reported detection limits of available commercial systems for detection of *Staphylococcus aureus* enterotoxins (SE) range from 0.5 to 2 ng enterotoxin per g of food (11, 16, 18, 20, 21). The sensitivity of detection methods and their ability to deliver quantitative information concerning the amounts of toxin present may require improvement, as it is possible that the established intoxication dose is underestimated due to constraining detection limits. Immuno-PCR is a detection method that combines the specificity of antibody-antigen recognition and the sensitivity of PCR. Immuno-PCR methods using endpoint detection with classical PCR and electrophoresis of amplicons for detection of *Clostridium botulinum* neurotoxin type A and E have been described (9, 10, 22). The immunoquantitative PCR (iqPCR) technology previously described in a patent (24) couples an antibody detection step, similar to an enzyme-linked immunosorbent assay (ELISA), with nucleic acid amplification of a DNA probe linked to the detection antibody by real-time PCR. Use of real-time PCR for quantitative amplification (13, 14), unlike endpoint analysis, provides data required for quantification of the target DNA. The results can be expressed in absolute terms by reference to an external quantified standard or in relative terms by reference to another target sequence present in the sample (19).

In the present work an iqPCR method for detection of *S. aureus* enterotoxin B (SEB) was developed and evaluated using both pure cultures and foods. The sensitivity of the iqPCR method was compared to the sensitivities of two commercially available systems (SET-RPLA [Oxoid, Basingstoke, United Kingdom] and VIDAS-SET2 [bioMérieux, Marcy-l'Etoile, France]), as well as to the sensitivity of an in-house ELISA that served as an internal reference for iqPCR.

* Corresponding author. Mailing address: Coupure links 653, B-9000 Ghent, Belgium. Phone: 32 9 264 61 78. Fax: 32 9 225 55 10. E-mail: mieke.uyttendaele@UGent.be.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains used (Table 1) were stored at −80°C and were resuscitated in tryptone soya broth (TSB) (Oxoid, Basingstoke, United Kingdom) by incubation for 48 h at 37°C. The purity of strains was checked and the reference stock cultures were maintained on tryptone soya agar (Oxoid) slants.

Enumeration of pure cultures and determination of *S. aureus* counts in sterilized food samples (with background flora eliminated) used for monitoring *S. aureus* growth and SE production were done by a plate spread method on tryptone soya agar (Oxoid) incubated at 37°C for 24 h. Presumptive *S. aureus* and total aerobic mesophilic counts for presumably naturally contaminated foods were determined on Baird Parker agar with egg yolk and tellurite (Merck, Darmstadt, Germany) and on plate count agar (Oxoid) using the classical plate count method, respectively.

Antibodies, antigens, and in-house ELISA. Five different sandwich formats using capture and detection antibodies and three direct ELISA formats using only a detection antibody were tested (Table 2). The formats were tested in duplicate and compared with each other using a twofold serially diluted standard solution of SEB (Toxin Technology, Inc., Sarasota, Fla.) as an antigen. All ELISA reactions were performed using 50 μl per well (96-well flat-bottom Microlon 600 extra-high-binding-capacity microtiter plates or Microlon 600 high-binding-capacity strip plates; Greiner bio-one B.V., Wemmel, Belgium), as described elsewhere (8).

iqPCR, primers, and DNA. To increase the sensitivity obtained with immunological detection in ELISA, a real-time iqPCR was developed using the same capture-detection antibodies (format 4) (Table 2). The ability to monitor the real-time progress of the PCR allowed quantification of 5′ biotinylated reporter double-stranded DNA bound to the biotinylated detection antibody via streptavidin, as shown in Fig. 1, using SYBR green sequence nonspecific chemistry (14). The 246-bp chimeric reporter double-stranded DNA (EMBL accession number AX133313) used was constructed by association of two DNA fragments of eukaryotic and prokaryotic origin (13, 14, 24), which decreased the risk of nonspecific amplification. The primers were designed with the Primer Express software (version 1.0; Applied Biosystems, Foster City, Calif.). The sequence of the forward primer was 5′-AAGCCTTGCAGGACATCTTCA-3′, and the sequence of the reverse primer was 5′-GCCGCCAGTGTGATGGATAT-3′. The

TABLE 1. Characteristics of *S. aureus* strains used in the present study

Strain	SE produced[a]	Other SE genes reported and possibly expressed[b]
ATCC 14458[c]	SEB	None
SK64f[d]	SEB	None
ATCC 13565[d]	SEA	*sed*, *sej*
ATCC 19095[d]	SEC	*seg*, *seh*, *sei*
ATCC 23235[d]	SED	*sei*, *sej*, *seg*
ATCC 27664[d]	SEE	None
171[e]	None (SEA to SEE)	None

[a] Classified by the *American Type Culture Collection* (*ATCC*) *Bacteria and Bacteriophage Reference Guide* (3).
[b] Strains harbored genes (4), but actual toxin expression was not confirmed due to the limitations of SET-RPLA and VIDA-SET2, which are not equipped with corresponding antibodies.
[c] Obtained from American Type Culture Collection.
[d] Gift from K. Cudjoe, Department of Feed and Food Hygiene, Section of Feed and Food Microbiology, Norwegian National Veterinary Institute, Oslo, Norway.
[e] Food isolate from the culture collection of Laboratory of Food Microbiology and Food Preservation, Ghent University, used as a negative control strain for classical enterotoxins, including SEA to SEE, and giving negative reactions in both SET-RPLA and VIDAS-SET2 assays.

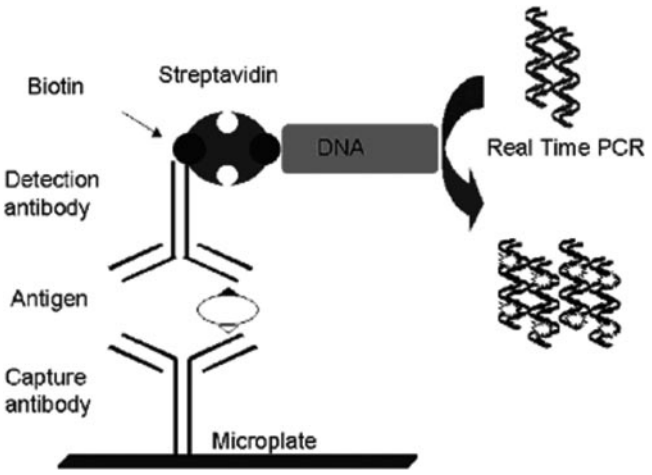

FIG. 1. Schematic diagram of the sandwich iqPCR model using the same capture and detection antibody for recognition of different epitopes on an antigenic molecule. Reprinted from reference 14 with permission from the American Association for Clinical Chemistry.

size of the amplicons obtained was 67 bp. iqPCR was carried out in Robostrips (Roboscreen, Leipzig, Germany) coated with immunoglobulin G-SLBI202 and saturated with optimized buffer. As in ELISA, the reaction volume was 50 μl, and the wells were prepared identically up to the point of addition of the biotinylated detection antibody (immunoglobulin G-SBBC202). After 1 h of incubation with the detection antibody at room temperature, the strips were washed three times with phosphate-buffered saline (PBS) (Sigma-Aldrich, Bornem, Belgium) containing 1 ml liter^{-1} Tween 20 (Sigma-Aldrich) and three times with PBS containing 15 g liter^{-1} bovine serum albumin (Sigma-Aldrich). Briefly, recombinant streptavidin (Roche, Vilvoorde, Belgium) diluted 1/500,000 was incubated for 45 min on ice with biotinylated reporter DNA (10 ng ml^{-1}) at a streptavidin/reporter DNA molar ratio of 1:2 (14). The resulting streptavidin-DNA complex was added to the wells and incubated for 30 min at room temperature. The strips were washed five times with PBS and 10 times with distilled water and were then subjected to PCR (ABI GeneAmp 5700 sequence detection system; Applied Biosystems, Foster City, Calif.) under the following conditions: 25 μl of SYBR green PCR Master Mix 2X (Applied Biosystems, Lennik, Belgium), 0.3 μM forward primer, 0.3 μM reverse primer, and 19 μl of water (total volume, 50 μl). The temperature program was as follows: 10 min at 95°C, followed by 40 PCR cycles of denaturation at 95°C for 15 s and 60°C for 1 min for the annealing and extension phases. The external standard (known concentration of DNA) was used at concentrations ranging from 2 pg liter^{-1} to 2 fg liter^{-1}. One no-template control contained the real-time PCR Master Mix without reporter DNA. The four other negative controls were samples without capture antibody, without antigen, without detection antibody, and without streptavidin-DNA complex. Amplification curves were analyzed with the Sequence Detection System software (version 1.9.1; Applied Biosystems, Foster City, Calif.), and the baseline was determined to circumvent background signals. The parameter threshold cycle (C_t), defined as the cycle number at which the fluorescence passed the fixed threshold, was used for quantitative determination of the initial SEB concentration. The cutoff value was adjusted manually on a run-to-run basis in order to select the best C_t value for each run (by selecting the parallel exponential phases for the different samples) and to be rigorous for the iqPCR comparison (sensitivity study).

A standard curve was produced by plotting the average C_t values of three repetitions against known SEB concentrations, and the linear range was used for

TABLE 2. Overview of ELISA formats tested, antibodies, and resulting sensitivities

Format	Coating material (capture antibody or antigen)	Detection antibody	Sensitivity (ng ml^{-1})
1	Mouse monoclonal antibodies, clone S222 (2S4) at 5 μg ml^{-1}[a]	Mouse monoclonal antibodies (biotinylated), clone S643 (2S4B) at 0.2–1 μg ml^{-1}[a]	NR[c]
2		Sheep polyclonal antibodies (biotinylated) (SBBC202) at 0.5 μg ml^{-1}[b]	~1.5
3	Sheep polyclonal antibodies at 10 μg ml^{-1} (SLBI202)[b]	Mouse monoclonal antibodies (biotinylated), clone S643 (2S4B) at 0.2–1 μg ml^{-1}[a]	NR
4		Sheep polyclonal antibodies (biotinylated) (SBBC202) at 0.5 μg ml^{-1}[b]	~1.5
5		Sheep polyclonal antibodies (POD conjugate) (SLBC202) at 3 μg ml^{-1}[b]	~7.5
6	Direct coating of SEB (BT202) (first well, 50 μg ml^{-1})[b]	Mouse monoclonal antibodies (biotinylated), clone S643 (2S4B) at 0.2–1 μg ml^{-1}[a]	NR
7		Sheep polyclonal antibodies (biotinylated) (SBBC202) at 0.5 μg ml^{-1}[b]	~12
8		Sheep polyclonal antibodies (POD conjugate) (SLBC202) at 3 μg ml^{-1}[b]	~97

[a] The code in parentheses is the internal code of the producer (HyTest, Ltd., Turku, Finland).
[b] The code in parentheses is the internal code of the producer (Toxin Technology, Inc., Sarasota, Fla.).
[c] NR, no reaction observed.

SEB quantification in the samples tested. To cover a wide detection range with the iqPCR, the SEB concentrations used were formulated in a fivefold dilution series. However, to match some of the SEB concentrations tested with the ELISA, an additional twofold dilution series was made in the linear range from 19.5 to 0.6 ng ml^{-1}.

The standard curve was reproduced using the same conditions in a different laboratory and with a different cycler (ABI Prism SDS 7000; Applied Biosystems, Foster City, Calif.).

The preliminary investigations of iqPCR specificity comprised a limited experimental setup with filter-sterilized cultures of *S. aureus* strains producing SE other than SEB (Table 1), as well as with cultures of a nonenterotoxigenic strain (which did not produce SEA, SEB, SEC, SED, and SEE), which were inoculated into TSB (approximately 6 log CFU ml^{-1}) and incubated for 24 h at 37°C.

Commercial SE detection systems. Staphylococcal enterotoxin test kits for detection of staphylococcal enterotoxins A, B, C, and D by reversed passive latex agglutination (SET-RPLA; Oxoid) and an enzyme-linked fluorescent assay (VIDAS-SET2) with a Mini VIDAS compact automated immunoassay analyzer (bioMérieux) for SEA, SEB, SEC, SED, and SEE were used for comparison and to confirm results obtained with iqPCR. The assays were performed according to the manufacturers' protocols.

Determination of SEB production in food and culture media. SEB production and detection in five different foods were investigated. Cooked ham, tuna, and paella were obtained from a local producer on the day of production. Additionally, milk reconstituted from a milk powder and a mixture of caramel and coffee creamer (mass ratio, 1:4) were both prepared in the laboratory. The pH and water activity of foods were measured using a pH meter (type 763; Knick, Berlin, Germany) with an electrode (model 104063123; Ingold, Urdorf, Switzerland) and a cryometer (AW-Kryometer; type AWK-20; NAGY Messsysteme GmbH, Gäufelden, Germany), respectively. Portions (50 g) of each food product were sterilized (121°C for 15 min), which ensured removal of the background flora and mimicked the processing conditions used for foods such as infant formulations and at the same time allowed unhampered *S. aureus* growth that would result in detectable SEB production. Sterilized foods were inoculated with 1 ml of an appropriate dilution of an overnight culture of an *S. aureus* strain (incubated at 37°C), providing a toxin-relevant inoculum level of approximately 6 log CFU g^{-1} or 6 log CFU ml^{-1}, and were incubated at the optimal *S. aureus* growth temperature (37°C) for 24 h. Two additional samples of milk were inoculated and incubated at 22 and 42°C for 24 h.

SEB was extracted from inoculated foods by mixing 10 g of food with VIDAS-SET2 extraction buffer (mass/volume ratio, 1:1). The mixture was incubated for 20 min at room temperature and centrifuged at 16,000 × *g* (22 min at 22°C). The supernatant was separated by injection through a syringe with prewetted cotton in its cylinder. The eluate collected was immediately analyzed for the presence of SEB (or stored for a maximum of 24 h at −20°C). All samples were analyzed by iqPCR, in-house ELISA, VIDAS-SET2, and SET-RPLA. Besides food samples, a standard solution of SEB spiked into TSB or milk was simultaneously examined by iqPCR, which allowed quantification of SEB. iqPCR measurement was performed in duplicate or triplicate. For VIDAS-SET2 SEB was extracted from another 10 g of each sample by following the manufacturer's instructions.

To understand the impact of different incubation temperatures on SEB production, two SEB-producing strains, *S. aureus* ATCC 14458 and SK64f, were inoculated into TSB at a concentration of approximately 2 log CFU ml^{-1}. Nonenterotoxigenic *S. aureus* strain 171 served as a negative control. Cultures were incubated at 10, 22, 37, and 42°C, and SEB production was analyzed after 4, 8, 12, and 24 h in duplicate. Analyses were performed using iqPCR and in-house ELISA.

SEB and microbiological analysis of presumably naturally contaminated foods. Six different, presumably naturally contaminated foods were obtained at the point of sale or directly from the producer on day 0 (strawberry-vanilla ice cream, peach fruit yoghurt, chocolate soy milk, ready-to-eat salmon-based salad, ready-to-eat tuna-based salad, and cooked ham). Each of the foods was divided into two portions (20 and 30 g). The 20-g portions were then divided into two 10-g portions, and therefrom two 8-g portions were spiked with 2 ml pure SEB (1 ng g^{-1}) to obtain a final concentration of 0.2 ng g^{-1}. Both 10-g portions (8 g food and 2 ml SEB solution) were extracted as described above and analyzed by iqPCR and VIDAS-SET2. The 30-g portion was used to determine the microbiological loads of the food (namely, the aerobic mesophilic count and the presumptive *S. aureus* count, as described above).

Statistical analyses. Statistical calculations of mean values, standard deviations, and 95% confidence intervals were performed with Microsoft Excel 2003. The normality of data distribution for both in-house ELISA and iqPCR was determined with SPSS 12.0 (SPSS, Inc., Chicago, IL) using the Shapiro-Wilks W test. Appropriate Pearson correlations between the values in the linear range for iqPCR (SEB concentration versus C_t) and for in-house ELISA (SEB concentration versus optical density) were calculated.

RESULTS

Development of an in-house ELISA and commercial assays. Although ELISA formats 2 and 4 had the same sensitivity, our choice to use format 4 throughout this study was based on the polyclonal recognition of multiple epitopes, which probably made it more tolerant to possible small changes in the nature of the toxin molecule induced during food preparation, thereby offering a more robust reactivity profile compared with monoclonal format 2. Figure 2 shows average ELISA results expressed as the optical density at 450 nm as a function of the SEB concentration obtained with pure SEB spiked into TSB (detection limit, approximately 1.5 ng ml^{-1}). The same sensitivity was obtained with milk spiked with SEB. SET-RPLA and VIDAS-SET2 detected SEB in the standard solution, spiked TSB, and spiked reconstituted milk powder (same samples that were used for ELISA) down to concentrations of approximately 0.6 and 0.15 ng ml^{-1}, respectively.

Using the antibodies described above with ELISA format 4, no signal greater than the noise (background plus 3 standard deviations) with strains producing SEA, SEC, SED, and SEE (filtrates of cultures incubated 24 h at 37°C) was observed.

Development of iqPCR. Detection of SEB with iqPCR started at a C_t greater than 20 (Fig. 3), allowing quantitative determination in the linear C_t range from approximately 23 to 30. Average measurements obtained with iqPCR with standard SEB spiked into TSB showed that the sensitivity was approximately 1,000 times higher (less than 10 pg ml^{-1}) than the sensitivity of ELISA (Fig. 2). Known concentrations of SEB spiked into reconstituted milk powder (starting concentration, 750 ng ml^{-1}) were detectable with iqPCR in a 10^5-fold-diluted sample (corresponding to approximately 7.5 pg ml^{-1}).

A comparison of data in the linear range for iqPCR with data from the in-house ELISA showed a high negative correlation, −0.947 (Pearson correlation; $P < 0.01$). The distribution of data was assumed to be normal as a prior Shapiro-Wilks test did not prove otherwise either for ELISA (W = 0.908; $P = 0.421$, N = 6) or for iqPCR (W = 0.951; $P = 0.750$; $n = 6$). This explains the linear association between the results in the linear range for these two tests.

In iqPCR, all samples tested containing SE other than SEB had a C_t value of about 30 and thus were comparable to the negative control sample. Nor were positive results observed in the SET-RPLA test with anti-SEB antibodies. This limited study indicated that there was no cross-reactivity of the SEB antibodies used with other SE or metabolites formed.

Determination of SEB production in food and laboratory media. All five foods inoculated with *S. aureus* had pH and water activity values greater than 5.8 and 0.98, respectively.

In paella and cooked ham inoculated *S. aureus* ATCC 14458 grew to concentrations of approximately 9 log CFU g^{-1}. In samples of the caramel-coffee creamer mixture and tuna no growth of *S. aureus* occurred. The resulting SEB production, as detected with iqPCR, is shown in Fig. 4. Quantitative interpretation of the data shown in Fig. 4 (based on a comparison of C_t values for different dilutions of food samples with C_t values for known SEB concentrations in TSB run in the same reaction)

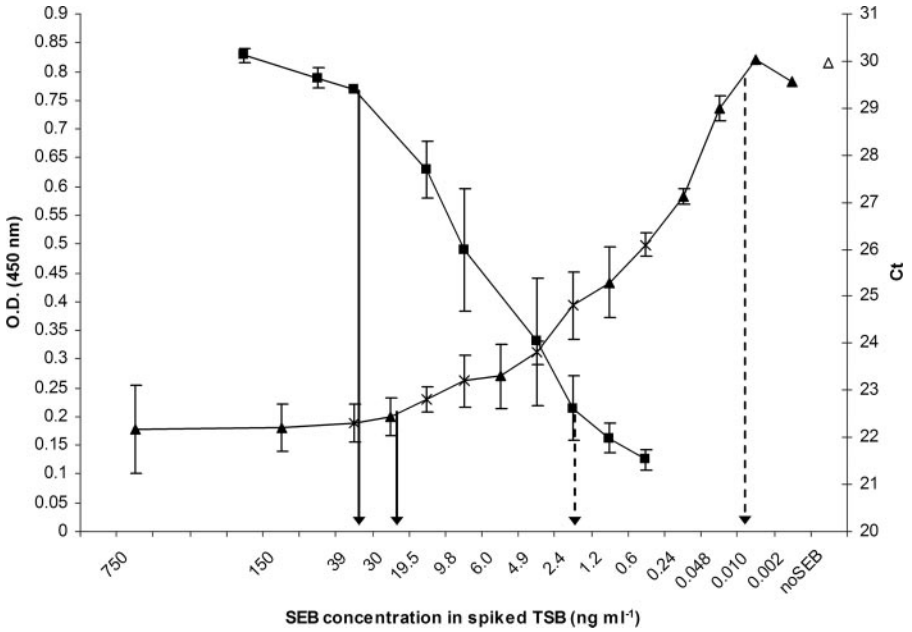

FIG. 2. Standard curves for ELISA format 4 with twofold serial dilutions of SEB solution in TSB (■), expressed as optical density at 450 nm [O.D.(450 nm)], and for iqPCR with fivefold (▲) and additional twofold (×) serial dilutions of SEB solution in TSB, expressed as C_t values, for detection of SEB spiked in TSB, showing the detection ranges and sensitivities of the two detection techniques. The dashed arrows indicate the start of the dynamic (quantitative) range for iqPCR and ELISA, and the solid arrows indicate the end of the dynamic range. A sample with no SEB (△) was used as a negative control in iqPCR.

showed that the amounts of SEB produced in paella and in cooked ham were 15 ng g^{-1} and 6 ng g^{-1}, respectively. SEB was detectable in the caramel-coffee creamer mixture and tuna, but the amounts were below the quantification limits, indicating that may have been transfer of SEB with the inoculum. Also, the VIDAS-SET2 analysis of inoculated tuna and the caramel-coffee creamer mixture showed a weak but positive reaction. The same samples were negative (no SEB detected) when they were tested with an in-house ELISA. Additional iqPCR performed with tuna and the caramel-coffee creamer mixture spiked with pure SEB (750 ng ml^{-1}) showed positive reactions until the concentration was 48 pg ml^{-1}, indicating that PCRs were not inhibited by the food matrix. Negative controls (food inoculated with *S. aureus* 171) and blank controls (noninoculated foods) were negative at all times.

Production of SEB at 22°C and 42°C compared to production at the optimal growth temperature (37°C) was tested further in milk. At all three temperatures *S. aureus* counts of approximately 7 log CFU ml^{-1} were obtained, resulting in production of approximately 75 ng ml^{-1} SEB (Fig. 5). No significant statistical difference at the 95% confidence interval was observed for SEB production and growth at the three temperatures tested.

Two *S. aureus* SEB-producing strains (ATCC 14458 and SK64f) were inoculated into TSB (approximately 2 log CFU

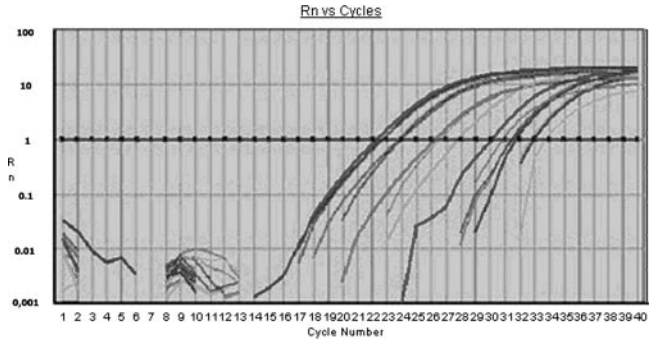

FIG. 3. iqPCR amplification plot for fivefold serial dilutions of SEB spiked into TSB (the starting and final SEB concentrations were 750 ng ml^{-1} and 1.8 pg ml^{-1}, respectively), showing the reporter signal versus amplification cycle. The four negative controls were a sample without SEB, a sample without coating antibody, a sample without detection antibody, and a sample without streptavidin-DNA complex; all had a C_t of more than 30.

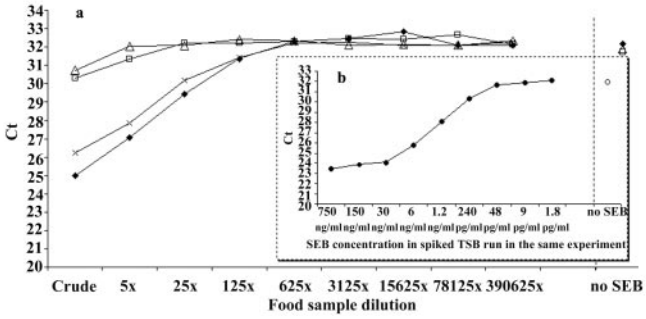

FIG. 4. (a) iqPCR results showing SEB production in paella (◆), cooked ham (×), tuna (△), and caramel-coffee creamer mixture (□), expressed as C_t values as a function of sample dilution. Noninoculated food samples served as a control without SEB. (b) Standard curves for pure SEB spiked into TSB (●), used for SEB quantification, and for PBS–1% bovine serum albumin without SEB (○), used as a control.

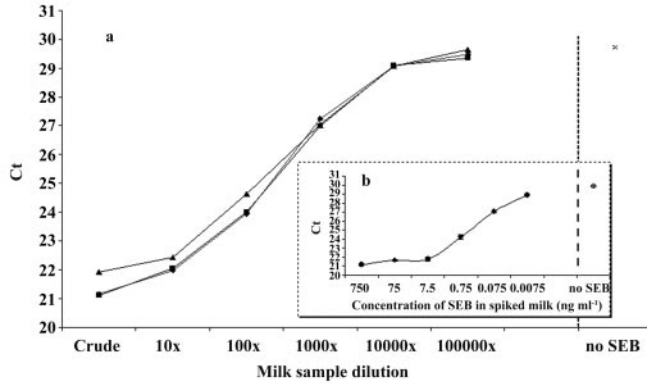

FIG. 5. (a) iqPCR results showing SEB production in milk at 22°C (◆), 37°C (■), and 42°C (▲), expressed as C_t values as a function of sample dilution. A noninoculated milk sample (×) served as a control. (b) Standard curves obtained with SEB spiked into milk (●), used for sample quantification, and for a milk control without spiked SEB (○).

ml^{-1}) and incubated at 10, 22, 37, and 42°C. Neither of the strains tested produced SEB at 10°C, and the number of cells of both strains remained at the inoculum level. Figure 6 shows the results of enumerations and analyses of SEB production at 22, 37, and 42°C. Samples that showed negative results in the ELISA (concentration lower than approximately 1.5 ng ml^{-1}) were retested with the more sensitive iqPCR. SEB production was found in all samples at 4 h. In general, the amounts detected at all temperatures were higher for *S. aureus* ATCC 14458 than for *S. aureus* SK64f. The amounts detected in TSB were higher at 42°C than at 37°C for both strains.

SEB and microbiological analysis of presumably naturally contaminated foods. iqPCR analysis of presumably naturally contaminated foods showed no detectable SEB in any of the foods tested except chocolate soy milk. All of the nonspiked foods except chocolate soy milk had C_t values higher than 31, similar to the values for negative controls (Fig. 7). Samples spiked with 0.2 ng SEB g^{-1} had C_t values between 27 and 28. A C_t value of 28 corresponds to an SEB concentration of approximately 0.12 ng g^{-1} (deduced from Fig. 2), indicating a possible minor loss of spiked SEB during extraction. Nonspiked foods were negative in the VIDAS-SET2 assay.

Microbial analysis showed that no *S. aureus* was present in any of the foods tested with an overall total aerobic mesophilic count of less than 10^3 CFU g^{-1}, except yoghurt samples, where the later count was approximately 10^7 CFU g^{-1}.

DISCUSSION

A large-scale outbreak in Japan involving more than 13,000 notified cases indicated that in different final prod-

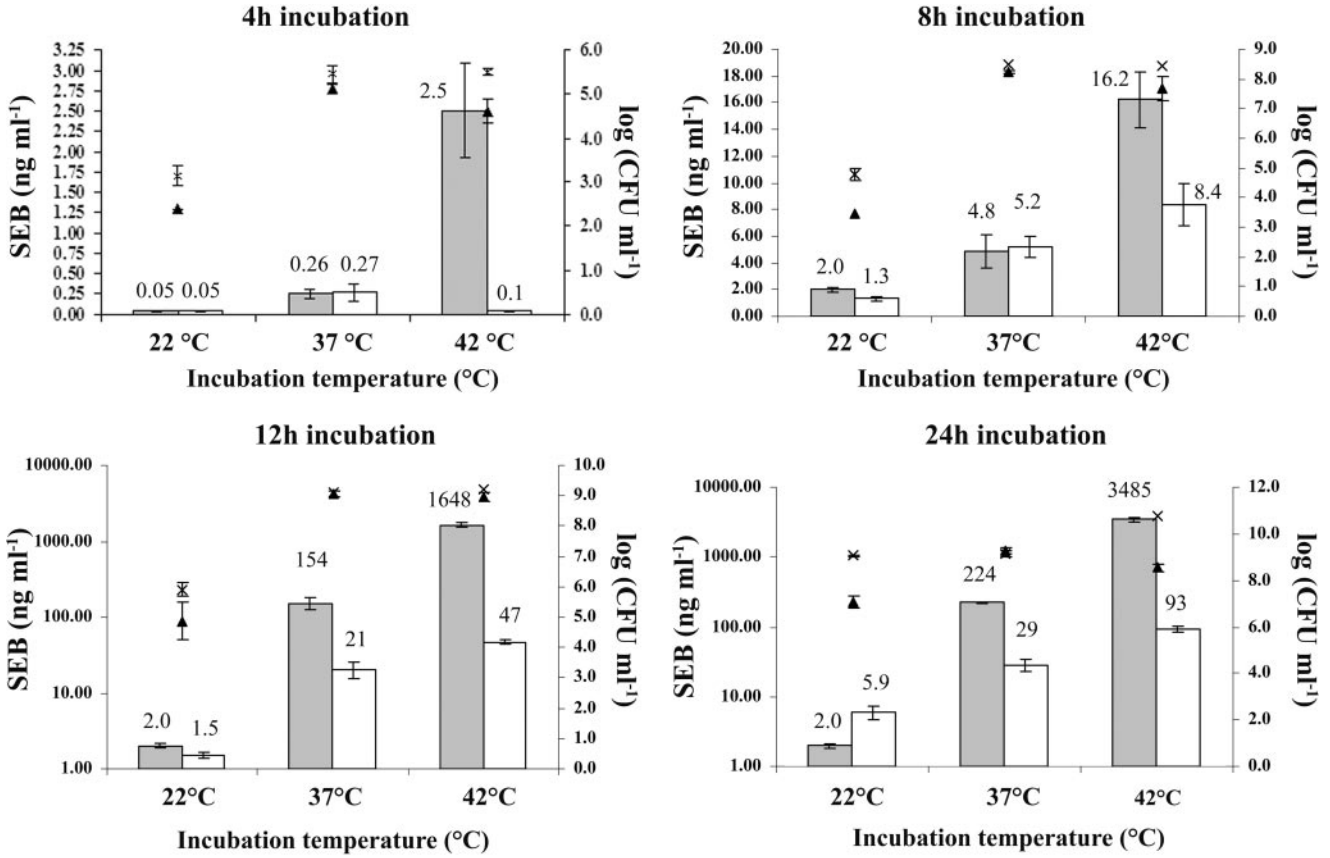

FIG. 6. Influence of incubation time and temperature on growth of (symbols) and SEB production by (bars) *S. aureus* ATCC 14458 (▲ and gray bars) and *S. aureus* SK64f (× and open bars). The error bars indicate standard deviations ($n = 2$) for average measurements of *S. aureus* counts and SEB concentrations. SEB production was determined by ELISA or by iqPCR for samples where ELISA gave negative results.

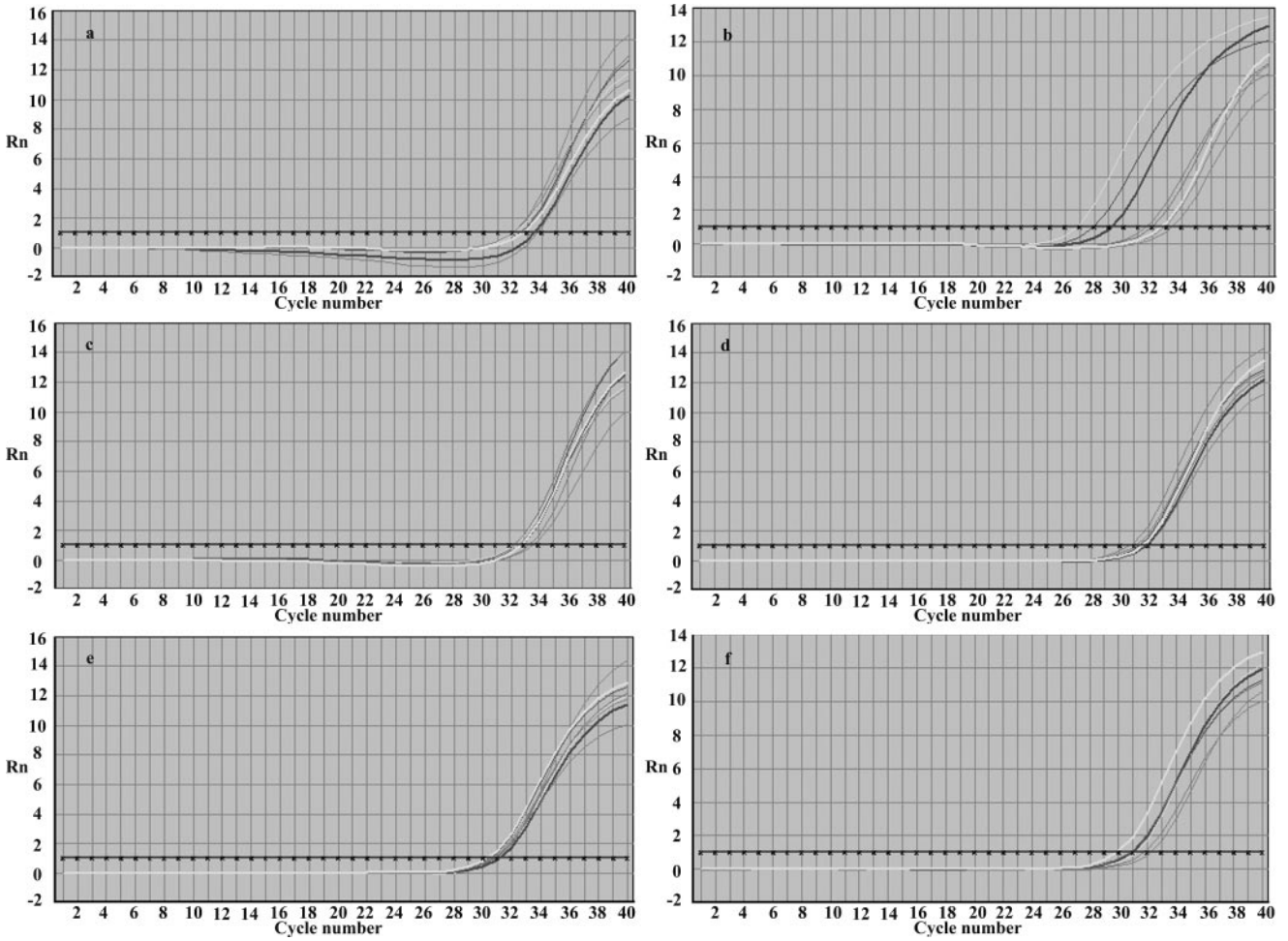

FIG. 7. Robustness and application of iqPCR in screening of presumably naturally contaminated foods for the presence of SEB. (a) Strawberry-vanilla ice cream; (b) chocolate soy milk; (c) ready-to-eat tuna salad; (d) peach fruit yoghurt; (e) ready-to-eat salmon salad; (f) cooked ham.

ucts 0.05 to 1.6 ng ml^{-1} of SEA was present, corresponding to approximately 4 ng g^{-1} of milk powder used to produce the final products (1, 2). However, *S. aureus* cells were not isolated from any of the samples obtained in this outbreak. This suggests that there is a need for reconsideration of conventional analyses, which focus mainly on detection of *S. aureus*, and that there is a the need for development of a more sensitive method for enterotoxin detection. The total amount of enterotoxins needed for intoxication was reported to be approximately 200 ng (12). The lowest concentration of SEB identified was 0.5 ng g^{-1} or 0.5 ng ml^{-1} (7, 20). These concentrations are detectable with VIDAS-SET2, which has a detection limit of less than 0.5 ng ml^{-1} (20), or by immunomagnetic flow cytometric detection of SEB, which has a detection limit of 0.25 ng ml^{-1} (17). However, the sensitivities of the methods that have been described flank the minimum intoxication dose, indicating that it is possible that the actual intoxication dose is lower but so far has not been detectable.

The real-time iqPCR used in the present study was highly sensitive, detecting SEB at concentrations of less than 10 pg ml^{-1}. The iqPCR described utilizes the same couple of polyclonal antibodies as a sandwich ELISA that was used for internal comparison, which has a detection limit of approximately 1.5 ng ml^{-1}. The dynamic range of iqPCR was C_t values of approximately 23 to 30, providing a quantification range of approximately 10 pg ml^{-1} to 30,000 pg ml^{-1}. Quantification could not commence at lower C_t values as the ROX signal of the real-time cycler was calibrated to start from C_t 7 to 15 (values near 13 in the current setup). A C_t value of more than 30 for the control sample containing no SEB was expected due to the minor cross-reactivity of the antibodies, allowing fixation of some DNA in the well and causing a consequent background signal.

The specificity of iqPCR proved to be satisfactory as none of the test strains producing SEA, SEC, SED, and SEE reacted positively in the iqPCR. Extensive work of Becker et al. (4) revealed that the most of the strains tested in the present study harbor genes encoding other SE too. These authors found that strain ATCC 13565 was also positive for SED production and for the presence of the *sej* gene; strain ATCC 19095 harbored the *seg*, *she*, and *sei* genes; and strain ATCC 23235 harbored the *seg*, *sei*, and *sej* genes. Assuming that there was expression of all these genes, no cross-reactivity in iqPCR was observed with any of enterotoxins (SEA to SHE).

The high specificity, high sensitivity, and wide quantification range enabled study of SEB production correlated with the growth status, extrinsic and intrinsic factors, such as type of food and storage temperature, and the *S. aureus* strain involved. Use of iqPCR for detection of SEB in early growth phases revealed the onset of SEB production after 4 h of incubation at 22, 37, and 42°C, which was in the first half of the exponential growth phase. Some other findings suggest that SEB production is related to the late exponential phase and the transition to the stationary phase (6, 15, 23), which can be the case for some other members of the SE family. A significant increase in SEB production was noticed when the *S. aureus* counts reached 5 log CFU ml^{-1}. At 37 and 42°C this level occurred after 4 h of incubation, while at 22°C the time required was more than 8 h. Incubation for 24 h at 10°C did not result in measurable SEB production, while at room temperature (22°C) 4 h was long enough to produce amounts reported in a recent outbreak (1, 2), corresponding to *S. aureus* counts of approximately 3 log CFU ml^{-1}. This is especially true when the background flora is absent or hampered in its competition with *S. aureus*, as shown in the present study with heat-treated foods. A temperature of 42°C favored SEB production compared to 22 and 37°C.

Since heat treatment of foods prior to inoculation with *S. aureus* eliminated the effect of the naturally present background flora, additional analyses of foods in their natural states were performed. iqPCR analysis of these foods showed that none of the foods tested inhibited PCR or impaired antigen-antibody recognition. In addition, iqPCR showed the presence of detectable amounts of SEB in one of the samples tested, as confirmed with VIDAS-SET2. Earlier consumption of the product from the same batch of chocolate soy milk caused complaints by consumers. High C_t values obtained with foods not spiked with SEB showed that no detectable amounts of SEB were present in them. This limited robustness test indicates that iqPCR performed well with different food matrices even with background flora present.

In this study iqPCR showed the potential for detection and quantification of small amounts of SEB in both pure cultures and food samples. To our knowledge, this is the most sensitive technique for detection of *S. aureus* enterotoxin B that has been reported so far. Further research will have to prove the relevance of the low detection limit with regard to the amounts of SEB found in food and other samples and their relationship to the exact intoxication dose and dynamics at which SEB is produced under the influence of different factors. Similar applications for other members of the SE family are foreseen, as is possible screening for enterotoxin production by other staphylococcal species, such as *S. intermedius* (5).

ACKNOWLEDGMENTS

This work was supported by Special Research Funds of Ghent University (Bijzonder Onderzoeksfonds, BOF) through a grant to A.R. for his Ph.D. studies.

We express our gratitude to K. Cudjoe from the Norwegian National Veterinary Institute for providing *S. aureus* strains and R. Reiser from Toxin Technology, Inc., for his valuable advice and support.

REFERENCES

1. **Anonymous.** 2001. *Staphylococcus* food poisoning in Japan. Infect. Agents Surveill. Rep. **22:**185–186. [Online.] http://idsc.nih.go.jp/iasr/22/258/tpc258.html.
2. **Asao, T., Y. Kumeda, T. Kawai, T. Shibata, H. Oda, K. Haruki, H. Nakazawa, and S. Kozaki.** 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.
3. **ATCC.** 1996. American Type Culture Collection (ATCC) bacteria and bacteriophage reference guide, 19th ed. American Type Culture Collection, Manassas, Va.
4. **Becker, K., A. W. Friedrich, G. Lubritz, M. Weilert, G. Peters, and C. von Eiff.** 2003. Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. J. Clin. Microbiol. **41:**1434–1439.
5. **Becker, K., B. Keller, C. von Eiff, M. Bruck, G. Lubritz, J. Etienne, and G. Peters.** 2001. Enterotoxigenic potential of *Staphylococcus intermedius*. Appl. Environ. Microbiol. **67:**5551–5557.
6. **Bennett, R. W.** 2005. Staphylococcal enterotoxin and its rapid identification in foods by enzyme-linked immunosorbent assay-based methodology. J. Food Prot. **68:**1264–1270.
7. **Bergdoll, M. S.** 1989. *Staphylococcus aureus*, p. 463–523. *In* M. Doyle (ed.), Foodborne bacterial pathogens. Marcel Dekker, New York, N.Y.
8. **Caponi, L., and P. Migliorini.** 1999. ELISA assays, p. 9–31. *In* L. Caponi and P. Migliorini (ed.), Antibody usage in the lab. Springer-Verlag, Berlin, Germany.
9. **Chao, H. Y., Y. C. Wang, S. S. Tang, and H. W. Liu.** 2004. A highly sensitive immuno-polymerase chain reaction assay for *Clostridium botulinum* neurotoxin type A. Toxicon **43:**27–34.
10. **Chao, H. Y., J. J. Wey, S. S. Tang, and A. Chen.** 2004. A highly sensitive immuno-polymerase chain reaction assay for *Clostridium botulinum* neurotoxin type E. Toxicol. Appl. Pharmacol. **197:**268.
11. **Di Pinto, A., V. T. Forte, G. Ciccarese, M. C. Conversano, and G. M. Tantillo.** 2004. Comparison of reverse passive latex agglutination test and immunoblotting for detection of staphylococcal enterotoxin A and B. J. Food Saf. **24:**231–238.
12. **Evenson, M. L., M. W. Hinds, R. S. Bernstein, and M. S. Bergdoll.** 1988. Estimation of human dose of staphylococcal enterotoxin-A from a large outbreak of staphylococcal food poisoning involving chocolate milk. Int. J. Food Microbiol. **7:**311–316.
13. **Gofflot, S., B. F. El Moualij, D. Zorzi, L. Melen, S. Roels, D. Quatpers, J. Grassi, E. Vanopdenbosch, E. Heinen, and W. Zorzi.** 2004. Immuno-quantitative polymerase chain reaction for detection and quantitation of prion protein. J. Immunoass. Immunochem. **25:**241–258.
14. **Gofflot, S., M. Deprez, B. el Moualij, A. Osman, J. F. Thonnart, O. Hougrand, E. Heinen, and W. Zorzi.** 2005. Immuno-quantitative PCR for prion protein detection in sporadic Creutzfeldt-Jakob disease. Clin. Chem. **51:**1605–1611.
15. **Klotz, M., S. Opper, K. Heeg, and S. Zimmermann.** 2003. Detection of *Staphylococcus aureus* enterotoxins A to D by real-time fluorescence PCR assay. J. Clin. Microbiol. **41:**4683–4687.
16. **Mathieu, A. M., B. K. Isigidi, and L. A. Devriese.** 1992. Comparison of 2 commercial kits for the detection of enterotoxins produced by *Staphylococcus aureus* strains isolated from foods. Lett. Appl. Microbiol. **14:**247–249.
17. **Miyamoto, T., H. Kamikado, H. Kobayashi, K. I. Honjoh, and M. Iio.** 2003. Immunomagnetic flow cytometric detection of staphylococcal enterotoxin B in raw and dry milk. J. Food Prot. **66:**1222–1226.
18. **Park, C. E., M. Akhtar, and M. K. Rayman.** 1994. Evaluation of a commercial enzyme-immunoassay kit (Ridascreen) for detection of staphylococcal enterotoxins A, B, C, D, and E in foods. Appl. Environ. Microbiol. **60:**677–681.
19. **Saunders, N. A.** 2004. Quantitative real-time PCR, p. 103–125. *In* K. Edwards, J. Logan, and N. A. Saunders (ed.), Real-time PCR: an essential guide. Horizon Bioscience, Norfolk, United Kingdom.
20. **Vernozy-Rozand, C., C. Mazuy-Cruchaudet, C. Bavai, and Y. Richard.** 2004. Comparison of three immunological methods for detecting staphylococcal enterotoxins from food. Lett. Appl. Microbiol. **39:**490–494.
21. **Wieneke, A. A.** 1991. Comparison of 4 kits for the detection of staphylococcal enterotoxin in foods from outbreaks of food poisoning. Int. J. Food Microbiol. **14:**305–312.
22. **Wu, H. C., Y. L. Huang, S. C. Lai, Y. Y. Huang, and M. F. Shalo.** 2001. Detection of *Clostridium botulinum* neurotoxin type A using immuno-PCR. Lett. Appl. Microbiol. **32:**321–325.
23. **Zhang, S. P., and G. C. Stewart.** 2000. Characterization of the promoter elements for the staphylococcal enterotoxin D gene. J. Bacteriol. **182:**2321–2325.
24. **Zorzi, W., B. El Moualij, D. Zorzi, E. Heinen, and L. Melen.** 2001. Detection method by PCR. Belgium patent WO 0131056.