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Detection of Enterotoxigenic *Staphylococcus aureus* in Dried Skimmed Milk: Use of the Polymerase Chain Reaction for Amplification and Detection of Staphylococcal Enterotoxin Genes *entB* and *entC1* and the Thermonuclease Gene *nuc*

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The polymerase chain reaction was used to amplify the staphylococcal enterotoxin B and C genes (*entB* and *entC1*) and the staphylococcal nuclease gene (*nuc*). Two sets of primers ("nested primers") were found to be necessary for the detection of low copy numbers of purified DNA in diluent. These allowed detection of ca. 1 fg of purified target DNA, while 100 pg was required before detection of *entB*, *entC1*, and *nuc* with single primer pairs was possible. With nested primers, enterotoxigenic *Staphylococcus aureus* cells could be detected in artificially contaminated dried skimmed milk samples at levels of ca. 10^5 CFU ml^{-1} within 8 h. No cross-reaction was observed between the highly homologous *entB* and *entC1* genes. The method showed total specificity for *entC1* when tested against a wide variety of other bacteria.

The mastitic udder is the most important animal source of staphylococcal contamination which may contain enterotoxigenic staphylococci other than *Staphylococcus aureus*. Staphylococcal enterotoxin C (SEC) is the enterotoxin most commonly associated with bovine, ovine, and caprine dairy produce (8).

S. aureus has consistently been one of the three most important microorganisms responsible for food-borne disease in the United States (13). Food-associated intoxications are commonly mediated by heat-stable staphylococcal enterotoxins (SEs) A to E. These are capable of surviving extreme conditions which may kill the staphylococcal cells. Staphylococci other than *S. aureus* have been shown to be potential enterotoxin producers. These include *S. hyicus*, *S. chromogenes*, *S. intermedius*, *S. saprophyticus*, *S. lentus*, *S. warneri*, and *S. sciuri* (23). In addition, antibodies reactive to toxic shock syndrome toxin-1 have been reported in milk (22).

The isolation and identification of enterotoxigenic staphylococci are procedures which necessitate a skilled operator and may require 4 to 8 days to complete. This normally involves isolation of organisms on selective media such as Baird-Parker medium (2) or, for heavily contaminated samples, Schleifer and Kramer medium (18), followed by a series of metabolic tests which may be performed by multipoint inoculation or by more rapid methods such as API Staph (7). Enterotoxigenicity may be tested for by rapid and sensitive serological methods such as reversed passive latex agglutination and enzyme-linked immunosorbent assay which are available commercially, but both are subject to interference by food components and protein A (5, 16, 25, 26).

A number of situations in which these methods are inadequate may arise. SEs may be produced by species not previously recognized as being enterotoxigenic and which could escape normal screening by coagulase and thermonuclease (TNase) testing (7). It has been reported that staphylococci other than *S. aureus* are capable of enterotoxin production and that unknown enterotoxins exist (1). Also, nontypical strains which may be enterotoxigenic but coagulase and/or TNase negative despite being of known species may fail to be detected. In a study of staphylococci isolated from healthy goats (23), it was found that 74% of coagulase-positive staphylococci and 22% of coagulase-negative strains tested were enterotoxigenic. Microbiological tests for the presence of staphylococci may be negative while SEs persist because of their extreme heat stability. Chemical factors may mask the presence of enterotoxins in immunological tests, resulting in false-negative reactions, particularly at low concentrations of enterotoxin. Differences in the production of enterotoxins by *S. aureus* grown in natural substrate and in laboratory media have been reported (5). Such factors may give rise to misleading results during laboratory examinations of suspected enterotoxigenic samples isolated from foods if culture takes place in a different growth medium.

The polymerase chain reaction (PCR) (17) offers the possibility of specific amplification of the gene responsible for SEC production. This technique allows the detection of the potential for enterotoxin production by cells which may not elicit toxin when grown in laboratory conditions and will also detect the inherent capability to produce SEs in killed cells whose enterotoxin may have survived thermal processing. Enterotoxin producers which have not as yet produced SEs but may later do so can also be detected by this method. A disadvantage of detection of enterotoxin genes by PCR rather than detection of the enterotoxins produced is the possibility of false-positive reactions under certain circumstances.

The detection of low numbers of bacteria by PCR has been reported (15) from environmental samples (20, 21) and tissue samples (6). A report has also been made of PCR detection of *Shigella flexneri* from spiked lettuce samples (11). Recently, a study on PCR amplification of *Listeria monocytogenes* DNA from soft cheeses (24) found that detection of less than 10^3 cells 0.5 g^{-1} was not possible because of interfering factors in the food. These findings are similar to the results of the present study.

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This paper describes a method for the amplification and detection of the *entC1* gene coding for SEC which can be applied to dried skimmed milk samples, yielding results within a day. In addition, the genes coding for SEB and the diagnostically important TNase enzyme were amplified.

MATERIALS AND METHODS

Recovery of DNA for PCR target material. Two methods were used to recover DNA from bacterial cells: (i) enzymic lysis followed by phenol-chloroform extraction and (ii) boiling.

(i) Total genomic DNA was obtained from *S. aureus* NCTC 10655 (SEC) by phenol-chloroform extraction. Cells were cultured for 18 h in 500 ml of Tripticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) shaken at 150 rpm on a model R-2 rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 37°C. The cells were spun down for 10 min with an MSE Hi-Spin 21 centrifuge (MSE Scientific Instruments, Crawley, England) at 5,000 × *g* (4°C). The pellet was suspended in 20 ml of 10 mM Tris (Sigma). Lysostaphin (to 600 μg ml^{-1}; Sigma) and sodium dodecyl sulfate (to 2% [wt/vol]; Sigma) were added, and the cells were incubated for 2 h at 37°C. For DNA extraction, an equal volume of Tris-saturated phenol (BDH Chemical Co., Poole, England) was added to the entire cleared lysate. After being vortexed and allowed to stand for 10 min, the phenol phase was removed and the aqueous phase was reextracted. This was repeated before extraction with 1 volume of phenol-chloroform-isoamyl alcohol (24:24:1). The aqueous layer was extracted again with 1 volume of chloroform-isoamyl alcohol (24:1). DNA was precipitated with 1/10 volume of 3 M sodium acetate (BDH) and 2 volumes of ice-cold ethanol. Centrifugation was carried out at 8,000 × *g* (4°C) to pellet the DNA after precipitation for 2 h at −70°C. Vacuum desiccation was used to remove traces of ethanol, and DNA was suspended in sterile TE8 (10 mM Tris, 1 mM EDTA, pH 8.0) by gentle vortexing. Stock solutions of known DNA concentrations were prepared from DNA quantitated by spectrophotometry (12).

(ii) Once conditions for amplification had been established with a stock solution of purified DNA, a simpler and more rapid method was adopted to obtain target DNA from other samples subsequently analyzed. Sterile plastic loops were touched to SEC-producing *S. aureus* NCTC 10655 colonies on 18-h nutrient agar plates which had been grown at 37°C, and small inocula were placed in 600-μl PCR tubes containing 99.5 μl of reaction mix (described below). Tubes were heated to 99°C in a thermal cycler for 15 min to lyse cells before addition of 0.5 μl of AmpliTaq polymerase (Perkin-Elmer) and thermal cycling as described below. Although DNA could be amplified directly from such boiled lysates, the target DNA was extracted from them with Isogene (ILS Ltd., London, England) according to the manufacturer's instructions, with the intention of removing any materials which might interfere with amplification. The PCR mix was made up to three times its original volume with NaI (i.e., to 4 mol $liter^{-1}$) and chilled on ice for 5 min. DNA binder reagent was vortexed, and 10 μl was added to the lysate. The tubes were mixed by manual end-over-end rotation at 4°C for 10 min to ensure optimal binding of DNA. Bound DNA was pelleted by centrifugation for 5 min at high speed in a microcentrifuge (Hermle).

The pellets were washed three times with 200 μl of wash buffer (1 M Tris, pH 7.5, 1 M NaCl, 100 mM EDTA diluted 1:100 with 70% [vol/vol] ethanol) with gentle resuspension of binder by vortexing for 5 s each wash. All supernatants were removed after the final wash, and DNA was eluted with 20 μl of deionized water, vortexed, and centrifuged as described in the instructions of the Isogene kit. A second elution with a further 20 μl of water released 70 to 80% of the DNA, as ethanol inhibited solubilization of DNA in the first wash, which yielded only 10 to 15% of the bound DNA.

PCR amplification of *entB*, *entC1*, and *nuc* with single and nested primers. Two 25-bp DNA primers were selected from the published DNA sequence of *entC1* (4) to allow amplification of the entire 801-bp gene. These were synthesized by NICGENE (Belfast, Northern Ireland) with an Applied Biosystems model 380B nucleic acid synthesizer. Their sequences were as follows:

Primer 1, 5′-ATG AAT AAG AGT CGA TTT ATT TCA T-3′

Primer 2, 5′-TTA TCC ATT CTT TGT TGT AAG GTG G-3′

PCR mixes were made from master mixes of components from the GeneAmp kit (Perkin-Elmer, Beaconsfield, England) as described in the instruction sheet. Each reaction tube contained 53.5 μl of double-distilled sterile water, 10 μl of 10× reaction buffer (100 mM Tris, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin), 16 μl of dinucleoside triphosphate mixture (each dinucleoside triphosphate at 1.25 M), 5 μl of primer 1 (20 M), 5 μl of primer 2 (20 M), 10 μl of phenol-chloroform-extracted target DNA (*S. aureus* NCTC 10655), and 0.5 μl of AmpliTaq recombinant DNA polymerase. This mix was contained in a total volume of 100 μl.

Evaporation within the tube was prevented by the addition of a 100-μl liquid paraffin overlay. To inactivate any proteases which may have been present, the tubes were heated to 94°C for 5 min prior to the addition of AmpliTaq.

A microprocessor-controlled thermal cycler (Perkin-Elmer) was used for automated temperature control of the PCR. DNA was amplified through 50 cycles of denaturation, annealing, and polymerization. Initially, denaturation was performed at 94°C for 1 min and primer annealing and polymerization were carried out at 37, 45, and 50°C for 3 min to determine the most effective temperature for amplification. After this had been determined, a polymerization step was introduced at 72°C, the optimum temperature of *Taq* polymerase. Cycles of 60, 30, and 10 s at each temperature were assessed. The optimized thermal profile was 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s.

A second set of 25-base DNA primers, internal to the first set and allowing amplification of a 631-bp fragment of the *entC1* gene, was synthesized. The sequences of these were as follows:

Primer 3, 5′-ACA CCC AAC GTA TTA GCA GAG AGC C-3′

Primer 4, 5′-CCT GGT GCA GGC ATC ATA TCA TAC C-3′

The nested primer arrangement is illustrated in Fig. 1.

From a 20 μM stock solution, 5 μl of each primer was added to the master mix described above and a dilution series of phenol-chloroform-extracted *S. aureus* DNA was amplified as before. Rather than removing an aliquot from amplification with one primer pair for further amplification with the second primer pair, the reaction took place in the presence of both primer pairs for a total of 50 cycles.

The sequences of the staphylococcal *nuc* gene coding for TNase (19) and the *entB* gene coding for SEB (10) allowed primers to be designed and synthesized to enable their amplification. The sequences of these primers were as follows:

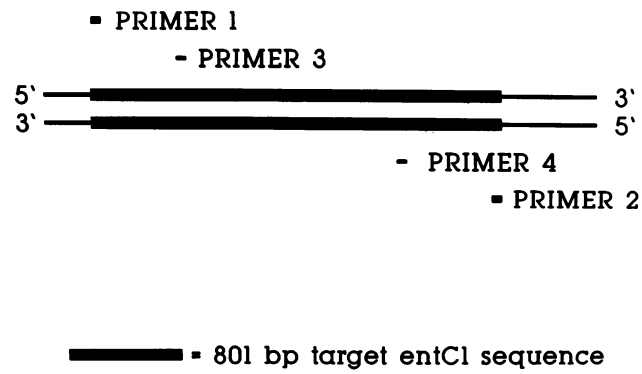

FIG. 1. Arrangement of nested primers for amplification of *entC1* gene by PCR. The wide bars show the 801-bp target *entC1* sequence.

TNase (*nuc*):

Primer 1, 5'-AGT ATA TAG TGC AAC TTC AAC TAA A-3'

Primer 2, 5'-ATC AGC GTT GTC TTC GCT CCA AAT A-3'

SEB (*entB*):

Primer 1, 5'-GAG AGT CAA CCA GAT CCT AAA CCA G-3'

Primer 2, 5'-ATA CCA AAA GCT ATT CTC ATT TTC T-3'

The *nuc* primers were used for amplification of a 450-bp sequence, and those for *entB* were used for a 593-bp fragment.

Detection of amplified DNA. A 15-μl aliquot of amplified DNA was examined after electrophoresis through a 1% Ultra-Pure agarose gel (BRL, Cambridge, England) in 1× Tris-borate buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0, [Kodak BufferEZE formula 3; Eastman Kodak, Liverpool, England]). After thorough mixing with tracking dye (40% [wt/vol] sucrose, 0.025% [wt/vol] bromophenol blue; Sigma) each sample was loaded into one well of the gel. Electrophoresis was carried out for 1 h at 80 V with a 3000/300 power pack (Bio-Rad).

The gel was removed from the chamber, immersed in an aqueous solution of 0.5 μg of ethidium bromide (Sigma) ml^{-1} for 20 min, and washed briefly in running tap water. The gel was examined visually with an Ultraviolet Transilluminator (UV Products, San Gabriel, Calif.) with a wavelength of 254 nm and photographed. The identities of the bands were established by molecular weight marker and DNA hybridization with a 39-bp oligonucleotide *entC1* probe (14).

Mg^{2+} concentration was optimized by titration with a series of 10× reaction buffers made up with the following final Mg^{2+} concentrations: 0, 10, 12, 14, 16, 18, 20, 30, and 40 mM. These buffers were identical to the 10× reaction buffer supplied with the GeneAmp kit (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl, 0.1% [wt/vol] gelatin), except that gelatin was excluded.

Specificity of amplification of *entC1* by PCR. The specificity of PCR was tested by inoculating small numbers of a wide variety of staphylococcal and nonstaphylococcal bacteria into reaction mixes, heating the mixtures to 99°C for 15 min, and then amplifying them for 50 cycles. These bacteria were *Escherichia coli* S128; *S. aureus* NCTC 10655 (SEC), NCTC 8532 (non-toxin producer), NCFB949 (non-toxin producer), NCTC 10652 (SEA), NCTC 10654 (SEB), NCTC 10656 (SED), ATCC 27664 (SEE), NCTC 10657 (SEA and SEB); *S. intermedius* NCTC 11048; *S. hyicus* NCTC 10350; *S. chromogenes* NCTC 10530; *S. saprophyticus* NCTC 7292; and *S. aureus* NF4001 (SEC) and NF4001 (non-toxin producer).

Sensitivity of detection of *S. aureus* from purified target DNA and from cell lysates. Decimal dilution series were prepared to investigate the sensitivity of detection from purified total genomic target DNA and from DNA released from dilutions of bacterial cells by boiling.

Control phage lambda DNA and associated primers supplied with the GeneAmp kit were used to determine the sensitivity of PCR with a DNA dilution series in TE8 as described in the instruction leaflet. The temperature profile used was 94°C for 5 min for the initial melting of DNA, followed by cycles of 94°C for 1 min (denaturation), 37°C for 1 min (primer annealing), and 72°C for 3 min (polymerization).

A similar decimal dilution series of phenol-chloroform-extracted DNA from SEC-producing *S. aureus* NCTC 10655 in TE8 and in sterile distilled water was amplified with primers for the *entC1* gene and the thermal profile 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. DNA was quantified spectrophotometrically (12).

SEC-producing *S. aureus* cells were inoculated from nutrient agar slopes and grown in shake flask cultures at 37°C for 18 h in nutrient broth; cells were spun down as described above, washed with quarter-strength Ringer solution (R/4), centrifuged, and resuspended in R/4; and a decimal dilution series was prepared in R/4. From each dilution, 10 μl was added to a reaction tube containing 90 μl of PCR mix, heated to 99°C for 15 min, and amplified for 50 cycles. Agarose gel electrophoresis was used to analyze the amplification products.

Detection of enterotoxigenic *S. aureus* in dried skimmed milk by PCR. Dried skimmed milk (1 g) was weighed aseptically into sterile 50-ml centrifuge tubes. To each of these was added 1 ml of serial dilutions from a 10^8-CFU ml^{-1} broth culture of *S. aureus* NCTC 10655 in sterile distilled water. Cell numbers were determined by plate count on nutrient agar after 24 h of incubation at 37°C.

Milk was solubilized by a method (9) which was originally developed to allow direct microscopic counts of bacteria in milk. To the 1-g sample inoculated with 1 ml of cell suspension, 35 ml of 0.015 N NaOH was added and roller incubated (Denley Spiramix) at 37°C for 1 h before centrifuging at 1,400 × *g* (20°C) for 15 min. The pellet was resuspended in 10 ml of 0.015 N NaOH and centrifuged as before to allow decanting of any milk not dissolved by the first treatment. The cell pellet was resuspended in 50 μl of TE8 and heated at 99°C for 15 min, and 10 μl of this was transferred to a PCR tube for amplification as described for purified DNA.

RESULTS AND DISCUSSION

Optimization of PCR conditions. Titration of the Mg^{2+} concentration of the reaction buffer revealed an optimum of 15 mM. Initially, primer annealing and polymerization were carried out for 3 min at 37, 45, and 50°C. These temperatures were arbitrarily chosen in relation to the low temperature of dissociation (T_d) of these sequences. Amplification of specific 801-bp PCR product for *entC1* was found only at 50°C, 3.5°C below the T_d of the lowest melting point primer as calculated by the following formula: (3) $T_d = 16.6(\log C_i) + 0.41[\%(G+C)] - 0.72$ (% formamide) $- 820/n - 1.5$(% mismatch), where C_i is the concentration of monovalent ions and n is the length of the hybrid (in base pairs). This was unexpected, as the reaction would be more likely to occur at the lower-specificity temperatures, which should have al-

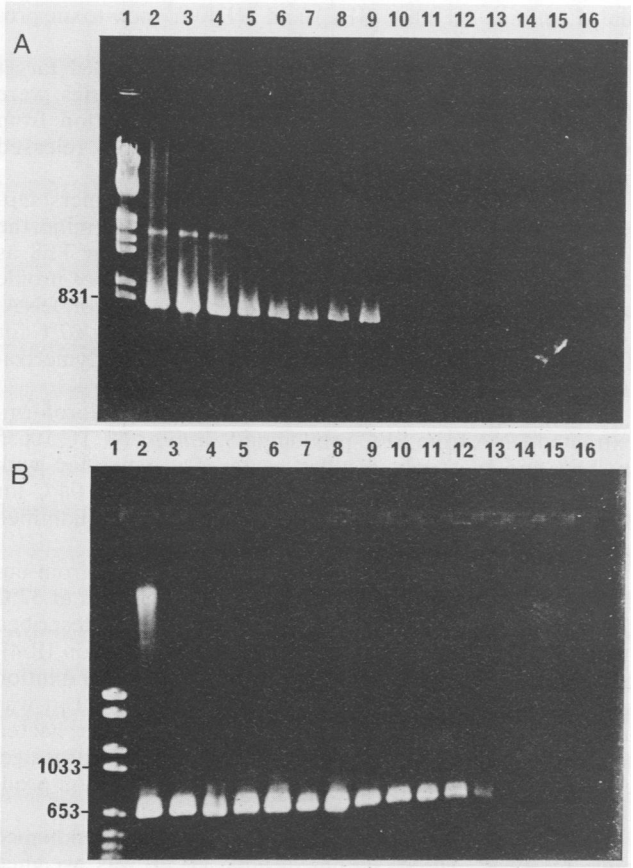

FIG. 2. Electrophoretic agarose gel analysis of PCR-amplified products from decimal dilution of *S. aureus* NCTC 10655 target DNA using primers 1 and 2 (A) and primers 3 and 4 nested within primers 1 and 2 (B) for the *entC1* gene. Lanes 1, DNA molecular weight markers lambda *Eco*RI digest (Sigma) (A) and VI (Boehringer-Mannheim) (B); lanes 2 to 16, decimal dilutions of total *S. aureus* NCTC 10655 DNA (1 mg to 0.01 fg). Sizes are marked in base pairs on the left.

lowed enough enzyme activity for the 801-bp fragment to be polymerized in 3 min. The finding may be explained by the binding of primers to nonspecific sites on the target DNA and low enzyme activity combining to produce small amounts of nonspecific reaction products of short length which were not detected on the gel.

Having determined an effective annealing temperature, the reaction profile was altered, as described in Materials and Methods, to allow the maximum amplification in the minimum time.

To improve enzyme efficiency, the temperature of polymerization was raised to 72°C, the manufacturer's stated optimum for *Taq* polymerase, and the optimal thermal profile was finalized as 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. This is shorter than most cycles reported in the literature and allows 50 cycles to be performed in 3 h.

Sensitivity of detection. Amplification of control lambda DNA showed that detection of 500 bp of PCR product from 100 fg of target DNA was possible. A decimal dilution series of phenol-chloroform-extracted *S. aureus* NCTC 10655 DNA showed that 100 pg of target DNA could be detected with single primers 1 and 2 for *entC1* and around 1 fg (equivalent to less than 10 cells) could be detected with

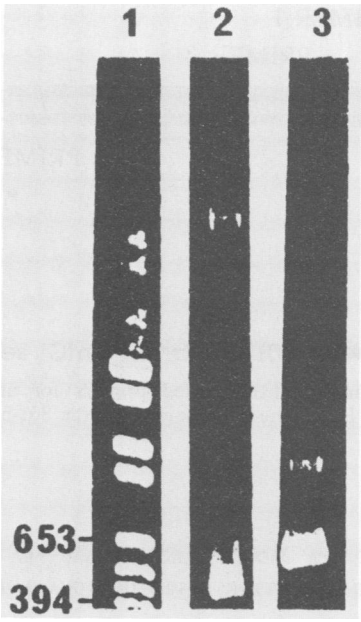

FIG. 3. PCR amplification of *entB* and *nuc* genes. Lane 1, DNA molecular weight marker VI (Boehringer-Mannheim); lane 2, amplified *nuc* gene (450 bp); lane 3, amplified *entB* gene (593 bp).

primers 3 and 4 nested internally within primers 1 and 2 (Fig. 2). Amplification of the *entB* and *nuc* fragments was accomplished at the same level of sensitivity (100 pg) as that of *entC1* with single primers (Fig. 3).

DNA released by heating SEC-producing *S. aureus* NCTC 10655 cells diluted in R/4 and sterile distilled water consistently allowed detection of 10^3 CFU per reaction tube (10^5 CFU ml^{-1}) with nested primers. Sensitivity was not increased by loading up to 50 μl of reaction product into each well of the gel or by amplifying for up to 100 cycles. Figure 4 shows a cell dilution series in R/4 with 50 μl of reaction product loaded per well (15 μl normally). Bands are sharp and unequivocal. Fifty cycles of amplification could be carried out in 3 h; electrophoresis and visualization required

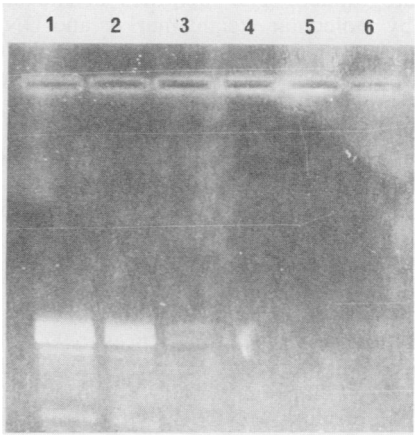

FIG. 4. PCR amplification of *entC1* gene from *S. aureus* NCTC 10655 cell dilution in R/4 by using nested primers. Lanes 1 to 6, decimal dilutions (10^5 to 10^0 CFU per reaction) of *S. aureus* NCTC 10655.

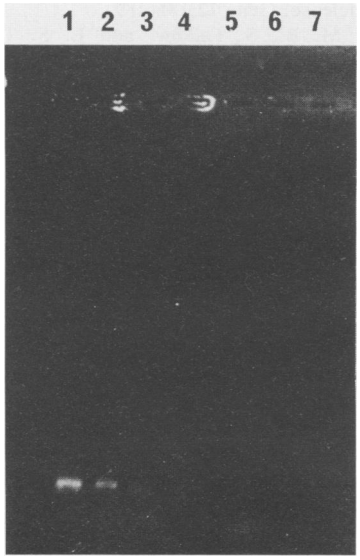

FIG. 5. PCR amplification of *entC1* gene from *S. aureus* NCTC 10655 cell dilution in dried skimmed milk by using nested primers. Lanes 1 to 7, decimal dilutions of Isogene-extracted total *S. aureus* NCTC 10655 DNA recovered from dried skimmed milk, 10^6 to 10^0 CFU per reaction.

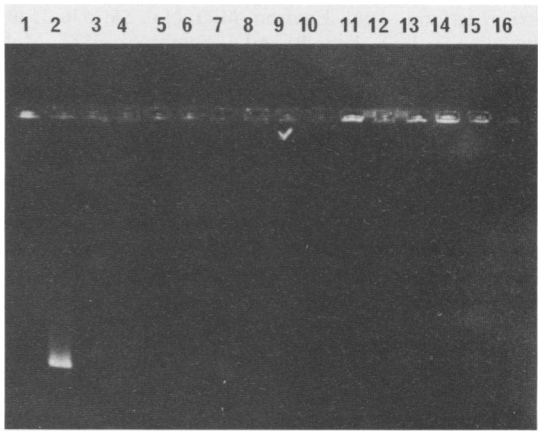

FIG. 6. Specificity of PCR amplification of *entC1* with nested primers. Lane 1, *E. coli* S128; lane 2, *S. aureus* NCTC 10655 (SEC) from dried skimmed milk; lane 3, *S. aureus* NCTC 8532; lane 4, *S. aureus* NCFB949; lane 5, *S. aureus* NCTC 10652 (SEA); lane 6, *S. aureus* NCTC 10654 (SEB); lane 7, *S. aureus* NCTC 10655 (SEC); lane 8, *S. aureus* NCTC 10656 (SED); lane 9, *S. aureus* ATCC 27664 (SEE); lane 10, *S. aureus* NCTC 10657 (SEA+B); lane 11, *S. intermedius* NCTC 11048; lane 12, *S. hyicus* NCTC 10350; lane 13, *S. chromogenes* NCTC 10530; lane 14, *S. saprophyticus* NCTC 7292; lane 15, *S. aureus* NF4011 (SEC); lane 16, *S. aureus* NF4001 (non-toxin producer). NF indicates our own laboratory isolates.

a further 1.5 to 2 h. This system therefore allows detection of ca. 10^3 enterotoxigenic cells (10^5 CFU ml^{-1}) in under 5 h. This is comparable with the detection of 10^3 enterotoxigenic *E. coli* cells reported (15) with primers for the heat-labile-toxin gene of this organism.

Figure 5 shows the amplification of *entC1* from *S. aureus* cells isolated from solubilized dried skimmed milk. Isogene extraction of DNA after lysis offered no improvement in sensitivity over simple heating and added over 1 h to the detection time. The detection limit was found to be around 10^3 to 10^4 CFU per reaction tube (10^5 to 10^6 CFU ml^{-1}). It is likely that Southern blotting of gels or DNA-DNA hybridization of the PCR product could allow an increase in sensitivity of 100- to 1,000-fold. This was not incorporated into the protocol because it would have added 2 to 4 days to the detection time, but situations in which sensitivity is considered more important than speed will exist.

Specificity of amplification. A 74% homology between *entC1* and *entB* was reported (4), but despite such similarities, Fig. 6 shows that nested primers for *entC1* allowed its detection with total discrimination from other SE genes and from the DNA of bacteria not producing these enterotoxins. In our laboratory, specificity proved superior to that of an oligonucleotide *entC1* probe (14) when this was labeled with digoxygenin rather than with ^{32}P as in the original report.

The findings reported here describe a rapid, sensitive, specific, and reliable method for the detection of enterotoxigenic *S. aureus* in milk. The fact that this technique allows detection of the genetic potential for enterotoxin production may make it useful as both a screening test and a confirmatory test for enterotoxins actually elicited, as determined by reversed passive latex agglutination or enzyme-linked immunosorbent assay. The data provided could yield additional information useful to epidemiological studies.

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