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Successful Approach for Detection of Low Numbers of Enterotoxigenic *Escherichia coli* in Minced Meat by Using the Polymerase Chain Reaction

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The polymerase chain reaction (PCR) was used as a tool for the detection of enterotoxigenic *Escherichia coli* in minced meat. With two synthetic 29-mer oligonucleotides, a 195-bp fragment from the *E. coli* heat-labile enterotoxin (LT) gene could be amplified specifically. When 6 CFU was added to the reaction mixture as a template, the PCR yielded sufficient amplified product for visualization on an agarose gel. Prior to PCR amplification, the minced meat samples were subjected to enrichment culturing for *E. coli*. From these cultures, 10 μl was used in the PCR assay. All 20 25-g samples that were examined in this assay were negative for *E. coli* LT. However, when 3 CFU of *E. coli* LT was added to the 25-g samples of minced meat prior to enrichment culturing, the PCR assay yielded positive results.

Enterotoxinogenic *Escherichia coli* (ETEC) strains are frequently associated with diarrheal disease in humans and animals (2, 4). These strains cause diarrhea by producing a heat-labile enterotoxin (LT), a heat-stable enterotoxin, or both. Infection from ETEC usually occurs as a result of the ingestion of contaminated food or water. Detection of pathogenic *E. coli* strains, including ETEC, is hampered by the lack of a specific culturing technique that allows direct discrimination from nonpathogenic *E. coli* strains. ETEC strains can be identified by detection of the enterotoxins in the culture fluid by immunological assays and bioassays (3, 14, 20). Alternatively, a DNA colony hybridization procedure can be used to recognize strains carrying the genes that encode the enterotoxins (1, 13, 16). Both options, however, require the identification of random *E. coli* isolates, making the techniques laborious and resulting in a low probability of finding a pathogenic *E. coli* isolate among a majority of nonpathogenic isolates. The recently developed polymerase chain reaction (PCR) technique (15) offers the possibility of reducing these drawbacks. By an in vitro procedure, specific gene fragments can be amplified enzymatically, allowing specific enrichment of the target DNA. Although numerous reports have described the use of the PCR for the detection of various pathogens in clinical settings, until now only a few reports have described the application of the PCR in food microbiology (5, 19), and none of these applications appears to be suited for routine investigations. At least three causes have contributed to this difference. First, the complex composition and wide variety of food matrices have hindered the development of a simple and universal procedure for the preparation of a DNA sample, allowing uninhibited PCR amplification. Second, the level of contamination with pathogenic microorganisms in food samples is usually far below that in clinical samples. Finally, food samples may contain pathogens which have already been killed during processing or remnants of their DNAs, leading to a positive PCR and to a false-positive diagnosis regarding the presence of a particular pathogen.

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In this report, we describe a procedure that meets the specific demands for the use of PCR in food microbiology and is suited for the routine investigation of minced meat for the presence of ETEC carrying the LT gene. It consists of a short enrichment followed by PCR amplification and yields a positive result even for samples with a very low ETEC contamination level. Moreover, the enrichment culture can be used directly in the PCR amplification assay without any pretreatment.

MATERIALS AND METHODS

Strains. All strains used in this study (see Table 1) were from culture collections at the National Institute for Public Health and Environmental Protection.

Culture conditions and enumeration techniques. Minced meat samples were obtained from local butcher shops and supermarkets. The microbial contamination of these samples was enumerated by various reference methods described by the International Organization for Standardization (ISO): ISO 2293 for the determination of the total aerobic count at 30°C, ISO 5552 for the enumeration of members of the family *Enterobacteriaceae*, and ISO 6391 for the enumeration of *E. coli* at 44°C with membranes.

The technique for enrichment of *E. coli* in the minced meat samples was a modification of the ISO 3811 method. A sample of 1 or 25 g was homogenized in 9 or 225 ml of lactose broth (5 g of peptone, 5 g of lactose, 3 g of Oxoid Lab-Lemco beef extract per liter), respectively. After 4 h of incubation in a 37°C water bath, brilliant green solution (100× concentrated) and ox bile solution (25× concentrated) were added to final concentrations of 0.0133 and 20 g/liter, respectively. This medium was designated LB. At the same time, the temperature was shifted to 44°C; incubation was then continued for 20 h. When required, a 0.1-ml sample was subsequently inoculated into 10 ml of brain heart infusion (BHI) broth and subcultured for 20 h at 37°C. Microbial counts were determined by the reference methods mentioned above.

DNA techniques. The two synthetic 29-mer oligonucleotides or amplimers that were used for the amplification of a

TABLE 1. Strains used in this study

Strain	Properties[a]
1. *Escherichia coli* H665/87	Clinical isolate; serotype O6K−; LT^+ and ST^+
2. *Escherichia coli* H92/88	Clinical isolate; serotype O6K15; LT^+ and ST^+
3. *Escherichia coli* H676/87	Clinical isolate; serotype O159K−; LT^+
4. *Escherichia coli* H104/87	Meat isolate; serotype O78K80; LT^+ and ST^+
5. *Escherichia coli* Brie	Soft cheese isolate; serotype O6K15; LT^+ and ST^+
6. *Escherichia coli* H664/87	Clinical isolate; serotype O99K−; ST^+
7. *Escherichia coli* H659/87	Clinical isolate; serotype O153K−; ST^+
8. *Escherichia coli* H658/87	Clinical isolate; serotype O153K−; ST^+
9. *Escherichia coli* H655/87	Food isolate; serotype O89K−; ST^+
10. *Escherichia coli* H661/87	Clinical isolate; serotype O27K−; ST^+
11. *Escherichia coli* H656/87	Clinical isolate; serotype O27K−; ST^+
12. *Escherichia coli* H19/WS	Clinical isolate; serotype O26K60; SLT^+
13. *Escherichia coli* H243/88	Clinical isolate; serotype O157K−; SLT^+
14. *Escherichia coli* H122/88	Clinical isolate; serotype O157K−; SLT^+
15. *Escherichia coli* EC1/E2	Meat isolate; nontoxinogenic
16. *Escherichia coli* EC8/D7	Meat isolate; nontoxinogenic
17. *Escherichia coli* EC1/A8	Meat isolate; nontoxinogenic
18. *Vibrio cholerae* El Tor	Food isolate; outbreak of cholera
19. *Yersinia enterocolitica*	Clinical isolate; case of food poisoning
20. *Yersinia pseudotuberculosis*	Clinical isolate
21. *Listeria monocytogenes* ScottA	Food isolate; serotype 4b; outbreak of food poisoning
22. *Klebsiella pneumotropica*	Fecal isolate
23. *Bacillus subtilis*	Soil isolate
24. *Bacillus cereus*	Milk isolate
25. *Micrococcus varians*	Water isolate
26. *Pseudomonas aeruginosa*	Clinical isolate
27. *Salmonella typhimurium*	Clinical isolate; case of salmonellosis
28. *Corynebacterium pyogenes*	Meat isolate
29. *Streptococcus faecium*	Fecal isolate
30. *Staphylococcus epidermidis*	Clinical isolate
31. *Staphylococcus aureus*	Food isolate; case of food poisoning
32. *Shigella flexneri*	Fecal isolate

[a] ST^+, heat-stable enterotoxin positive; SLT^+, Shiga-like toxin positive.

195-bp DNA fragment from the *E. coli* LT gene were based on the sequence for the B subunit of the LT gene of porcine origin (6). The nucleotide sequences of the left- and right-hand amplimers were 5′-CCTCTCTATATGCACACGGAG CTCCCCAG-3′ and 5′-CTATATGTTGACTGCCCGGGAC TTCGACC-3′, respectively. For identification of the amplified PCR product, an internal 20-mer probe with the sequence 5′-ATACGGAATCGATGGCAGGC-3′ was used.

PCR was performed essentially as described by Saiki et al. (15). The standard reaction mixtures (100 μl) contained 10 μl of test sample, 50 mM Tris HCl (pH 8.5), 50 mM NaCl, 1 mM $MgCl_2$, 2 mM dithiotreitol, 0.1% (wt/vol) Triton X-100, 50 μM each deoxynucleoside triphosphate, 0.2 μM each amplimer, and 2.5 U of Taq polymerase (Cetus). After being covered with 50 μl of paraffin oil, the reaction mixtures were heated to 94°C for 3 min. Then PCR was carried out in a programmable heating block incubator (New Brunswick Scientific) for 40 reaction cycles (one cycle: 1 min at 94°C followed by 3 min at 54°C and then 3 min at 72°C). Subsequently, 25 μl of the reaction mixtures was fractionated by electrophoresis on a 1.6% agarose gel, and the amplified DNA fragment was visualized by ethidium bromide staining and UV transillumination (at 302 nm) of the gel (7).

When required, the amplified LT gene fragment was identified by hybridization with the internal ^{32}P-labeled 20-mer probe. For this, the gel was blotted onto a Hybond-N membrane (Amersham) and hybridized with the radiolabeled probe as described previously (10). After hybridization, the membrane was washed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer at 60°C and autoradiographed.

RESULTS

PCR for *E. coli* LT. To demonstrate the functionality of the selected amplimers for the amplification of the 195-bp LT gene fragment, we tested various bacterial cultures (Table 1). As shown in Fig. 1, only the *E. coli* LT-positive (LT^+) strains yielded an amplified fragment of the predicted size. Although some other *E. coli* strains yielded some minor aspecific bands, these could not be mistaken for the LT gene fragment, since they had different molecular weights. This result indicates that the amplimers are suitable for the specific amplification of a fragment from the LT gene. The detection limit of the assay was determined by amplification of diluted samples from a pure *E. coli* LT^+ culture (strain H665/87). As shown in Fig. 2, the presence of 6 CFU in 10 μl of LB culture medium yielded sufficient product for visualization on an agarose gel.

Detection of *E. coli* LT in minced meat. Two samples of minced meat were used to investigate the feasibility of the PCR for the detection of *E. coli* LT. The initial microbial load of these samples was determined, and the samples were then subjected to the enrichment culturing described in Materials and Methods. As shown in Table 2, the total aerobic plate counts for the two minced meat samples were 4.0×10^5 and 1.8×10^7 microorganisms per g, whereas the number of *E. coli* was less than 10^2 CFU. Because of the selectivity of the enrichment procedure, the final LB culture contained about 10^4 CFU of *E. coli* per ml, approximately 0.1 to 1% of the total microbial plate count. After subsequent dilution and further incubation in BHI broth, the number of *E. coli* further increased to 10^8 to 10^9 CFU/ml.

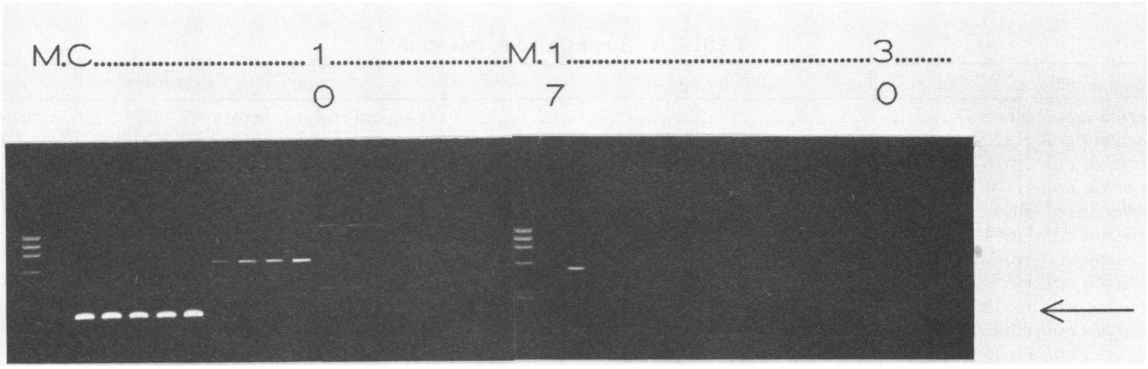

FIG. 1. Amplification of the 195-bp fragment of the *E. coli* LT gene from various strains. Lanes correspond to the strains listed in Table 1 (e.g., lane 10 is *E. coli* H661/87). The marker lanes (M) contain *Hae*III-digested φX174 DNA. The control lane (C) contains a sample without DNA. The arrow indicates the position of the 195-bp DNA fragment.

One-gram portions of minced meat were mock infected with 3, 10, 30, and 100 CFU of an *E. coli* LT^+ strain (H665/87) subjected to enrichment culturing, and assayed by PCR. Enrichment cultures to which no *E. coli* LT^+ strain was added all yielded negative PCR results throughout the entire enrichment procedure (Fig. 3). On the other hand, enrichment cultures of mock-infected samples were all positive, even when inoculated with only 3 CFU of the *E. coli* LT^+ strain. Gel analysis of the amplified samples revealed one prominent band of the predicted molecular weight. No significant effect of the initial *E. coli* LT^+ strain inoculation rate, the enrichment culture fluid used (LB or BHI broth), or 10-fold dilution of the samples prior to PCR amplification was seen (Fig. 3).

In the next experiment, the PCR assay was used to examine 20 lots of minced meat for the presence of *E. coli* LT. From each lot, 1- and 25-g samples were subjected to enrichment culturing and PCR. To check the integrity of the assay, we carried out parallel experiments in which an average of 3 CFU of an *E. coli* LT^+ strain was added to the meat samples prior to enrichment culturing. In four of these meat samples, the initial microbial load and the effect of enrichment culturing in LB were determined, with and without mock infection (Table 3). The corresponding gel analysis and Southern blot analysis of PCR amplification assays for the cultures containing 25 g of meat are shown in Fig. 4. In these amplification assays, 10 μl of an undiluted LB enrichment culture was used without any pretreatment. For all minced meat samples (both 1 and 25 g) examined, this quantity yielded a negative result. In all parallel assays with mock-infected minced meat samples, a positive result was obtained, except for one assay in which a 1-g minced meat sample was used. As shown in Fig. 4, the results from the gel analysis of the amplification assays were unambiguous. Subsequently, the PCR amplifications were carried out with 10 μl of an undiluted BHI enrichment culture, yielding results that were identical to those obtained with the corresponding LB cultures (data not shown).

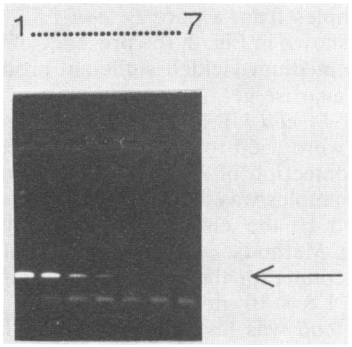

FIG. 2. Detection limit for *E. coli* LT in the PCR. Lanes 1 to 7 contain amplification mixtures to which 6×10^3, 6×10^2, 6×10^1, 6, 6×10^{-1}, 6×10^{-2}, and 0 CFU of strain H665/87 were added as templates, respectively. The arrow indicates the position of the 195-bp DNA fragment.

DISCUSSION

We describe here a procedure for the detection of *E. coli* LT in minced meat. It consists of a combination of enrichment culturing and PCR amplification of a DNA fragment of the LT gene. Under the established reaction conditions, the set of amplimers used yielded a specific amplification product of the expected size. When a pure *E. coli* LT^+ culture was used as a target, 6 CFU yielded sufficient amplified product for visualization on an ethidium bromide-stained agarose gel. This sensitivity is in agreement with those reported elsewhere for the detection of microorganisms by PCR (9, 12, 17).

The use of an enrichment culturing procedure prior to PCR amplification was shown to be advantageous for the examination of minced meat samples for the presence of *E.*

TABLE 2. Effect of the enrichment procedure on the bacterial counts determined for two minced meat samples

Meat sample and organisms	Count		
	Initial (CFU/g)	Final (CFU/ml) in:	
		LB	BHI broth
Beef			
Total	4.0×10^5	2.0×10^6	8.1×10^8
Enterobacteriaceae	2.0×10^4	7.0×10^5	8.0×10^8
E. coli	$<1.0 \times 10^2$	3.5×10^4	5.0×10^8
Beef and pork (mixed)			
Total	1.8×10^7	5.5×10^7	5.0×10^8
Enterobacteriaceae	5.5×10^4	3.4×10^7	4.4×10^8
E. coli	$<1.0 \times 10^2$	2.0×10^4	3.5×10^8

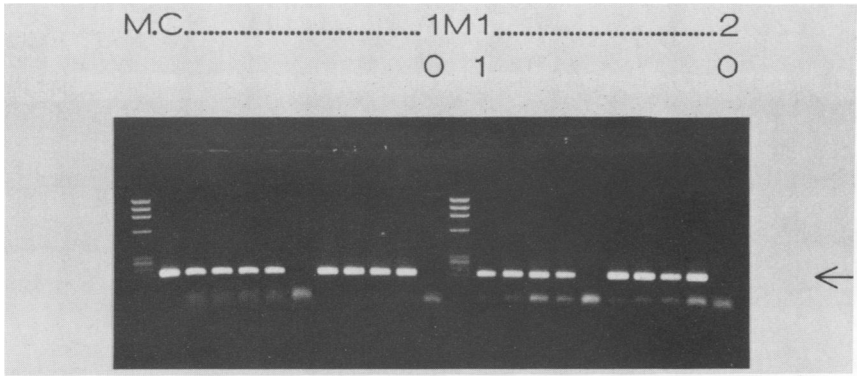

FIG. 3. Detection of *E. coli* LT in 1-g samples of minced meat mock infected with strain H665/87. Lanes: 1 to 5, samples mock infected with 100, 30, 10, 3, and 0 CFU, respectively, and amplified from a 10-fold-diluted LB culture; 6 to 10, like lanes 1 to 5, but amplified from an undiluted LB culture; 11 to 15, like lanes 1 to 5, but amplified from a 10-fold-diluted BHI broth culture; 16 to 20, like lanes 1 to 5, but amplified from an undiluted BHI broth culture. The control lane (C) was amplified from a pure culture of strain H665/87 (2×10^4 CFU). The marker lanes (M) contain *Hae*III-digested φX174 DNA. The arrow indicates the position of the 195-bp DNA fragment.

coli LT. In the six samples of minced meat that were examined in more detail in this study, an average total contamination level of 10^7 microorganisms per g was observed, whereas the number of indigenous *E. coli* organisms was below 10^2. For five of the six samples, the final LB enrichment culture contained 2.0×10^4 to 7.0×10^6 *E. coli* CFU/ml; one sample remained negative. However, when 3 CFU of an *E. coli* LT^+ strain was added to the latter sample prior to LB enrichment culturing, the final *E. coli* count was 2.4×10^7 CFU/ml. A comparison of these values with the total count and the number of *Enterobacteriaceae* organisms demonstrated the selectivity of the procedure for the growth of *E. coli*. After subculturing in BHI broth, even higher numbers of *E. coli* could be obtained.

In none of the minced meat samples was indigenous *E. coli* LT found. This observation is in agreement with the results of Notermans et al. (11), who used a colony hybridization procedure to examine minced meat for the presence of *E. coli* LT and obtained no positive results. In our experiments, mock infection of the samples resulted in a positive PCR result, except for one 1-g sample, whereas the corresponding 25-g sample was positive. This observation makes it unlikely that the negative result was due to an inhibitory effect of the PCR on the sample. A more probable explanation comes from a statistical analysis of the data. Assuming a Poisson distribution profile for the inoculation of 40 samples with an average of 3 CFU, it can be calculated statistically that 2 samples (5%) are expected to receive none (18). This distribution is likely to be the cause of the one negative PCR result.

TABLE 3. Effect of enrichment culturing in LB on the bacterial counts, with and without mock infection with an *E. coli* LT^+ strain (3 CFU), in four samples of minced meat (25 g)

Meat sample and organisms	Count		
	Initial (CFU/g)	Final (CFU/ml) in LB:	
		Without mock infection	With mock infection
1			
Total	1.2×10^7	3.0×10^5	3.5×10^7
Enterobacteriaceae	3.0×10^3	1.9×10^5	3.0×10^7
E. coli	$<1.0 \times 10^2$	$<1.0 \times 10^2$	2.4×10^7
2			
Total	3.6×10^6	3.6×10^6	2.1×10^6
Enterobacteriaceae	3.0×10^6	3.0×10^6	2.0×10^6
E. coli	$<1.0 \times 10^2$	1.1×10^6	1.4×10^6
3			
Total	1.4×10^7	8.5×10^6	5.7×10^7
Enterobacteriaceae	8.0×10^3	4.1×10^6	2.7×10^7
E. coli	$<1.0 \times 10^2$	4.5×10^6	3.0×10^7
4			
Total	7.4×10^6	7.2×10^6	4.5×10^7
Enterobacteriaceae	9.0×10^3	7.0×10^6	2.7×10^7
E. coli	$<1.0 \times 10^2$	7.0×10^6	3.0×10^7

The procedure described here uses a sample from an enrichment culture without any pretreatment prior to PCR amplification. From many other reports, it is known that the thermostable *Taq* polymerase in the PCR assay is very susceptible to inhibition by compounds that may be present in a sample (8, 12, 19). As a result, extraction and purification of the DNA from the sample prior to PCR may be needed to remove the inhibitory compounds. However, in earlier experiments on the use of the PCR for the detection of microorganisms in food products, we found that even this pretreatment may be insufficient. For this reason, we had to conclude that a PCR-based method for the direct detection of *Listeria monocytogenes* in soft cheeses would not be applicable for routine investigations (19). Surprisingly, in the method described here, the samples did not have a markedly negative effect on the results or reproducibility of the assay. Apparently, the composition of the samples did not interfere with the action of the *Taq* polymerase.

When the PCR assay is used as a diagnostic tool for the detection of microorganisms in food, the presence of microbial target cells already killed during processing may be a point of concern. Their DNAs may still be intact, yielding a positive PCR result. In the procedure described here, this risk was strongly reduced by enrichment culturing of the target cells that were alive and concomitant dilution of dead cells by subculturing. For an average of one dead cell to be present in the PCR assay with a BHI broth culture, 10^5 cells must be present per ml of a final LB enrichment culture.

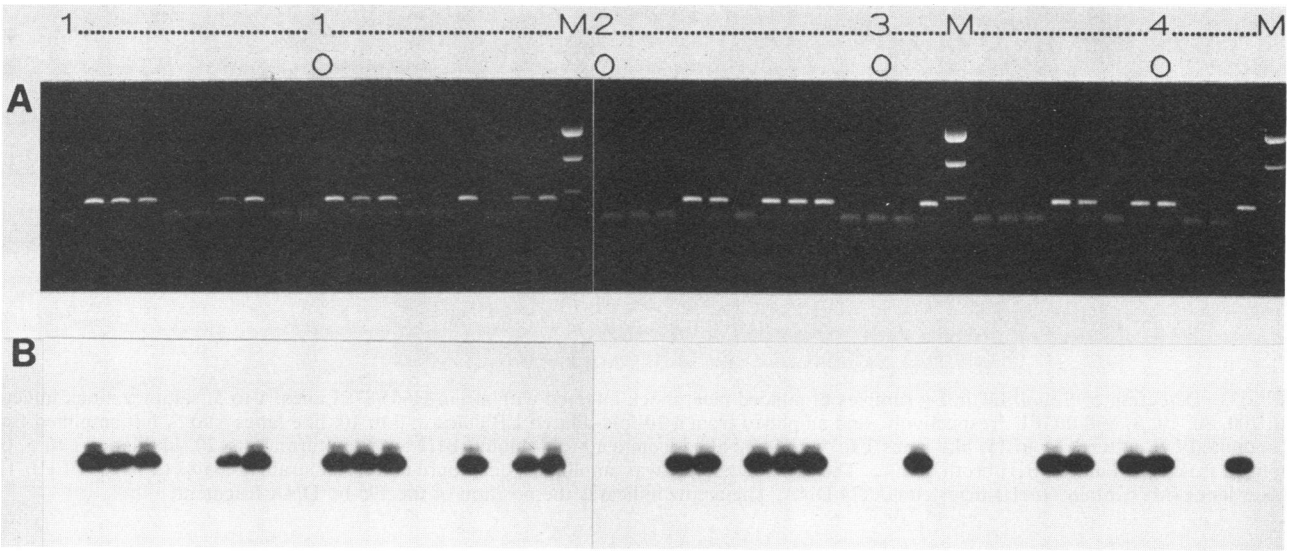

FIG. 4. PCR amplification of LB enrichment cultures containing 25 g of minced meat. Lanes 4, 7, 8, 11, 12, 13, 16, 18, 19, 23, 24, 26, 27, 28, 32, 36, 37, 39, 40, and 43 contain control samples mock infected with strain H665/87. Lanes 2 and 3 contain pure cultures of H665/87 diluted in LB and water, respectively. Lane 1 contains sterile distilled water. The marker lanes (M) contain *Rsa*I-digested pUC19 plasmid DNA. The remaining lanes contain samples without mock infection. (A) Gel analysis. (B) Southern blot analysis.

This means that 10^6 dead cells must be present per g of sample.

In the experiments described here, the amplimers were annealed to the target DNA at 54°C. At this temperature, satisfactory results were obtained with respect to the stringency of the amplification for the LT gene fragment. Although we observed some minor aspecific amplification products with pure cultures of various bacterial strains (Fig. 1), these could not be mistaken for the LT gene fragment, since they had different molecular weights. It should be mentioned, however, that 54°C, although satisfactory, may not be the optimum annealing temperature for the amplimers used in this study. Minor variations have been reported in the nucleotide sequences of various ETEC isolates of human or porcine origin (6). The sequences of the amplimers used here were based on the LT gene of porcine origin and compared with the sequences of the human *E. coli* LT^+ isolates; the left-hand amplimer contained two internal mismatches. Thus, these amplimers may be unsuitable for efficient amplification of the corresponding gene fragments from some ETEC isolates. This possible disadvantage can easily be circumvented by choosing the amplimers from an area in which the two genes show full homology.

We believe that the method described here meets the most important demands for use in a routine setting. It is simple and rapid, low numbers of *E. coli* LT^+ organisms can be detected reproducibly, and it is relatively insensitive to false-positive results due to the presence of killed target cells.

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