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Rapid and Specific Detection of Enterotoxigenic *Escherichia coli* and *Salmonella* Strains by Multiplex PCR Systems

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ABSTRACT

Escherichia coli and *Salmonella* are two of the most important food-borne bacterial pathogens. Classical identification for these strains is laborious, time-consuming, and may generate erroneous results. The purpose of this study was to develop a rapid and specific multiplex PCR (m-PCR) method to simultaneously detect heat labile enterotoxin gene of *E. coli* (LT ETEC) and o*riC* of *Salmonella* sp. Multiplex PCR using two pairs of primers produced specific amplicons of expected sizes of 163 bp and 425 bp from mixed populations of *Salmonella* sp. and LT ETEC bacterial strains, respectively. These primers were then used for the detection of food and feces with 10^1-10^2 cells/g of *Salmonella* and LT ETEC, followed by SCLB (selenite cystine-lactose broth, selenite cystine / lactose broth, 5/3, w/w) incubation. The presence of these two pathogens in food and feces was detectable. Finally, we used this method for the detection of 160 kinds of market-available foods and feces, and found that LT ETEC bacterial strains were detected in 2 samples (poultry and feces), and one sample (feces) by the BAM (Bacteriological Analytical Manu) method.

Key words: multiplex PCR, heat labile enterotoxin *E. coli*; *Salmonella*

INTRODUCTION

Escherichia coli and *Salmonella* are two major food-borne bacterial pathogens[1]. Many food-borne outbreaks of *E. coli* or *Salmonella* sp. have been associated with contaminated consumables, such as beef, pork, chicken and water[2]. The risk of human illness can be better predicated by monitoring microbial contamination at points of potential contamination during processing, distribution, or selling in retail markets[3]. As a result, contamination of pathogenic microorganism is recognized as a potential public health concern.

Conventional methods for detecting the presence of microbial pathogens are based on culture followed by a series of presumptive and confirmatory tests[4]. Traditionally, food-borne microorganisms are detected by selective media although a pre-enrichment step is required in some cases. This plating technique, based on the phenotype of bacteria, is labor-intensive and can take several days to confirm suspicious colonies by biochemical and serological tests. One obstacle to the study of diarrhea caused by *E. coli* is the differentiation of enterotoxigenic strains from normal flora. Although conventional methods, which are based on biological and immunological assays, can be used for the detection of enterotoxins[5], high cost and inconvenience make these methods unsuitable for large-scale studies. In order to reduce the time required, a rapid and accurate identification of bacterial pathogens from food samples is important, both for food quality assurance and to trace outbreaks of bacteria pathogens in food supply.

Polymerase chain reaction (PCR) technology has been proven an invaluable method for the detection of pathogens in food. PCR represents a rapid procedure with both high sensitivity and high specificity, allowing for almost immediate detection and identification of specific pathogenic bacteria in various food matrices and fecal culture[6,7]. In a few cases, multiplex PCR has been used to detect several pathogens in a single reaction[8,9]. Multiplex PCR systems were developed to detect *S. enterica* servoar Typhimurium and *S. enterica* servoar Eenteritidis[8], four major *E. coli* virotypes[10], five main pathotypes of diarrheagenic *E. coli* and *Shigella* spp.[11], the *ltI*, *stIa*, *stlI* and *vt2* genes[10,12], *lt* and *st*I[13], genes coding for fimbriae, enterotoxins, shiga toxins of *E. coli* isolates[14] and pathogenic *Salmonella* strains and *E. coli* O157:H7[1,3]. However, the ability to enrich several pathogens in enrichment broth, followed by a PCR protocol suitable to amplify and detect multiple templates, would extend our surveillance capability.

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In this study, specific primers, M1 and M2[15] which were published earlier by other researchers, were employed for multiplex PCR detection of *Salmonella ori*C gene. To meet the requirements for distinguishable amplified LT ETEC and *Salmonella* by multiplex PCR, it was necessary to select new primers within the target genes of LT ETEC. Two novel universal primers, LT1L and LT1R, which encode consensus sequences of enterotoxin genes, were selected by computer analysis of nucleotide sequences for the corresponding heat labile enterotoxin genes (GenBank accession number K01995). Two primer pairs, M1/ M2, LT1L / LT1R, were then used in multiplex PCR for the assay of culture mix of *Salmonella*, LT ETEC and various samples. Despite requiring separate enrichment of both pathogens, we used a new, less selective enrichment broth of SCLB (selenite cystine-lactose broth) to enrich both pathogens. Our results showed that multiplex PCR using the two primer sets was specific for the detection of *ori*C and heat labile enterotoxin genes.

MATERIALS AND METHODS

I. *Bacterial Strains*

The *E. coli* strains used in this study included: enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC) and nonpathogenic *E. coli*. A number of *Salmonella* sp., as well as *Shigella*, *Citrobacter*, *Klebsiella*, *Enterobacter* spp. *etc*. strains were also used (Table 1). These bacterial strains were obtained from the American Type Culture Collection (ATCC); the Center for Disease Control (CDC), Georgia, U.S.A., the United States Department of Agriculture (USDA), Washington, DC., World Health Organization (WHO), Washington, U. S. A., the City of New York Department of Health (US), U. S. A., Food and Drug Bureau, Department of Health Executive Yuan, Taiwan, R. O. C. (FDB), Bioresource Collection and Research Center (BCRC), Hsinchu, Taiwan; Bureau of Food and Drug Analysis (BFDA), Taipei, Taiwan; and Ping Tung University of Technology (PT), Pingtung, Taiwan. Some bacterial strains were laboratory isolates.

Bacteria cells were cultivated in Luria broth (tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g, in 1000 mL of dist water) overnight at 37°C with rotary shaking. Stock cultures were kept at -80°C in 20% glycerol.

II. *PCR Primers*

The novel PCR primers designed from the *E. coli* heat-labile enterotoxin gene (GenBank accession number K01995) were LT1L (5'-CAAACCGGCTTTGTCA GATAT-3') and LT1R (5'-ATGAATTTCCACAACCCTAT-3'), and produced a DNA fragment of 425 bp. Primers M1 (5'-TTATTAGGATCGCGCGAGGC-3') and M2 (5'- AAAGAATAACCGTTGTTCAC-3') were specific to the origin of replication on the *Salmonella* chromosome (o*riC*)[15].

III. *DNA Preparation and Multiplex PCR (m-PCR)*

For the PCR assay, the cell lysate was used as a source material, and the method of Tsen *et al*.[16] was modified for cell lysate preparation. In brief, 4 μL of the overnight culture cells were mixed with 196 μL of sterilized water for 10 min of boiling.

The reaction mixture contained 0.5 μg of genomic DNA, 2.5 units of *Taq* polymerase (Promega, Madision, WI, USA), 2 μL each of 10 mM dATP, dTTP, dCTP and dGTP, 5 μL of 10 X reaction buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C), 0.01% Triton X-100, 0.01% gelatin, 6.0 mM $MgCl_2$), and 50 pmol of each primer containing LT1L, LT1R, M1 and M2 in a final volume of 50 μL. The DNA was denatured at 94°C for 2 min and amplified for 35 cycles at 94°C for 40 sec, 55°C for 50 sec, and 72°C for 40 sec. A final extension incubation of 2 min at 72°C was included. Amplification reactions were performed on a thermal cycler (Perkin-Elmer GeneAmp PCR System 2400). The amplification products were loaded onto a 1.8% agarose gel. After electrophoresis in 1X TBE (Tris-Borate-EDTA) buffer at 50 volts, the gel was stained with ethidium bromide before being photographed by ultraviolet illumination.

IV. *Sensitivity for the Detection of Salmonella and LT ETEC*

Overnight cultures of *Salmonella* and LT ETEC were diluted with sterile water in decimal series. One milliliter of the diluted mixture containing *Salmonella* and LT ETEC was used for DNA extraction and PCR amplification with the procedures described above.

V. *PCR Detection of Salmonella and LT ETEC in Food Samples*

Food, water and feces were collected from local markets and from healthy students of Department of Food Science and Technology, Chia Nan University of Pharmacy and Science. Twenty five grams of minced food sample were mixed with 225 mL of 0.1% peptone water and homogenized. For evaluating the sensitivity for this method, various concentrations (0, 10^1-10^4 cells/mL) of each LT ETEC and *Salmonella* sp. were simultaneously added to the homogenate. To increase the sensitivity of detection, 1 mL of the sample mixture was mixed with 9 mL of lactose broth, and the mixture was incubated at 37°C and shaken for 8 hr. Then 1 mL of this culture was transferred to 9 mL of SCLB (selenite cystine broth / lactose broth, 5/3, w/w, combined with autoclaved lactose broth, 53.4 g/L and heated selenite cystine broth, 18.14 g/L) or performed with conventional methods of incubation at 37°C and shaking for 8-12 hr. All the samples

Table 1. Specificity of the multiplex PCR

Species	No. of isolates	*Positive results*		Species	No. of is1olates	*Positive results*	
		M1-M2	LTIR-LTIL			M1-M2	LTIR-LTIL
Acinetobacter calcoaceticus (ATCC 19606)	1	0	0	*S. anatum* (USDA807EI, US)	2	2	0
Alcaligenes faecalis (ATCC 8750)	1	0	0	*S. azteca* (PT 607)	1	1	0
Bacillus sutilis (ATCC 21778)	1	0	0	*S. bousso* (PT 643)	1	1	0
Brevibacterium linens (ATCC19391)	1	0	0	*S. derby* (CDC RF62)	1	1	0
Citrobacter freundii (ATCC 8090, 10787)	2	0	0	*S.enterica serovar* Enteritidis ATCC 13076, US)	2	2	0
Enterobacter aerogenes (ATCC 13048, US)	2	0	0	*S. essen* (PT 661)	1	1	0
Enterobacter cloacae (ATCC 23355	1	0	0	*S. hvittingfoss* (USDA)	1	1	0
Escherichia coli (ATCC 25922, 11775, FDB E01-E07, FDB E2416-E2422)	16	0	0	*S. limete* (PT 669)	1	1	0
E.coli (LT ETEC) ATCC 37218, 33849, WHO112, 117)	4	0	4	*S. London* (PT 1004)	1	1	0
E.coli (LT & ST ETEC) (ATCC35401, WHO 110)	2	0	2	*S .manhattan* (USDA 1007B1)	1	1	0
E. coli (EIEC) (ATCC 43983, BFDA 11096, 11098)	3	0	0	*S. Miami* (USDA)	1	1	0
E. coli (EHEC)	3	0	0	*S. nigor* (PT 695)	1	1	0
Klebsiella pneunoniae (BCRC 10692)	1	0	0	*S. partyphi A* (PT 398)	1	1	0
Serratia marcescens (SER10)	1	0	0	*S. sentftenberg* (USDA 1073C)	1	1	0
Shigella flexner (ATCC 12022, 29903)	2	0	0	*S. tennessee* (USDA 1258)	1	1	0
Salmonella				*S. typhi* (ATCC 8427)	1	1	0
S. aberdeen (US)	1	1	0	*S.enterica serovar* Typhimurium (ATCC 14028, 13311, USDA 1024)	3	3	0
S. Adelaide (US)	1	1	0	*S. worthington* (PT 658)	1	1	0
S. agona (PT 624)	1	1	0				

*The original sources of bacteria used in this study.

were analyzed by preparing DNA for the multiplex PCR as described above.

VI. *Conventional Method*

Methods as described by BAM[4] was used for *Salmonella* and LT ETEC detection. The samples from above in lactose broth were used and 1 mL was transferred to 9 mL of tetrathionate broth, and then was incubated at 37°C and shaken for 24 hr. Such culture was plated on *Salmonella-Shigella* agar (SSA) and then incubated at 37°C for 48 hr. The colony formed was trans-

ferred into triple sugar iron (TSI) agar and then incubated at 37°C for another 48 hr. Further biotest and serotyping tests were performed for the identification of *Salmonella* sp. For *E. coli* strains, 1 mL of the homogenous sample was inoculated into 9 mL of lauryl sulfate tryptose (LST) broth. After incubation at 35°C for 24-48 hr, gas production in tubes was observed. This method was called the BAM gas production method. To confirm the presence of *E. coli* cells, 1 loopful of the culture in each gassing LST tube was transferred to each of the 10 mL EC broth (*Escherichia coli* broth) and the mixture was incubated at 45.5°C for another 24-48 hr. Samples in gassing EC broth were streaked onto the Levine Eosin Methylene Blue agar (L-EMB) plate and incubated at 35°C for another 18-24 hr. These plates were inspected for the presence of presumptive *E. coli* colonies (the MPN method). The colonies were then streaked on PCA slants and subjected to IMViC confirmation test. The heat-labile enterotoxin (LT) production was examined by using the reversed passive latex agglutination kit (VET-RPLA, Denka Seiken, Tokyo, Japan).

RESULTS AND DISCUSSION

I. *Specificity of the Multiplex PCR (m-PCR) System*

To investigate specificity of this method, both non-LT ETEC and non- *Salmonella* sp. strains listed in Table 1 were employed as negative control, along with known LT ETEC and *Salmonella* sp. in the test. These negative reference strains included EHEC and EIEC strains that do not produce heat-labile enterotoxin and some isolates that commonly contaminate food.

The two pairs of the synthetic-specific oligonucleotide primers for multiplex PCR were termed LT1L/LT1R, M1/M2. Among them, M1, M2 were derived from Mahon *et al.*[15] which were specific for the origin of replication on the *Salmonella* chromosome (o*riC*) in PCR, while LT1L, LT1R encoded from the heat-labile toxin gene of *E. coli*, and were tested and found to have specificity as PCR primers for detecting the heat-labile toxin gene (data not shown). The DNA sequences of LT1L, LT1R primers differ from primers reported by other studies[16]. Our results showed that under the selected conditions as described in "Materials and Methods", the application for the detection of *Salmonella* and LT ETEC gene was specific. The relative molecular sizes of the PCR products amplified from *Salmonella* sp. and LT ETEC genes with multiple primers were 163 bp and 425 bp, respectively (Figure 1). This result was consistent with those predicted from the primer design. In contrast, none of the primer pairs reacted with any of the non-LT ETEC or non-*Salmonella* sp., such as: EIEC, EHEC, *Shigella*, *Citrobacter* and *Klebsiella* spp., etc. listed in Table 1. From the above results, specificity of the multiplex PCR system was confirmed.

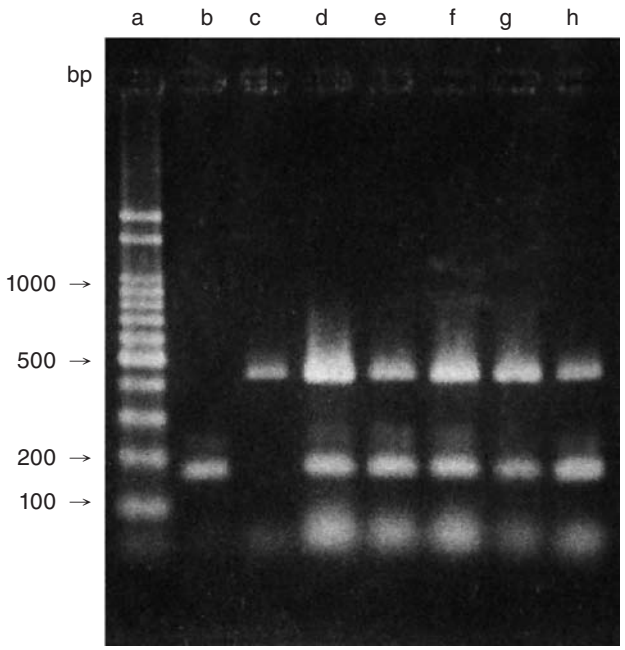

Figure 1. Specificity for detection of LT ETEC and *Salmonella* strains using multiplex PCR system. The PCR primers used were LT1L/LT1R and M1/M2. Lane a: 100 bp DNA ladder (Bertec Enterprise Co., Ltd. Taipei, Taiwan); Lane b: *S. enterica* serovar Typhimurium (BCRC12948); Lane c: LT1 ETEC (ATCC43886); Lane d: LT1 ETEC (ATCC43886) / *S. enterica* serovar Typhimurium (BCRC12947); Lane e: LT1ETEC (ATCC33849)/*S. typhi* (BCRC12947); Lane f: LT1h+ST1h ETEC (ATCC35401)/*S. enterica* serovar Enteritidis (ATCC13076); Lane g: LT1h+ST1 ETEC (WHO110) / S. bousso; Lane h: LT1ETEC (ATCC33849)/*S. adelaide*.

II. *Sensitivity of the m-PCR*

To test sensitivity of the assay, the PCR templates were prepared by mixing equal amounts of bacteria cultures followed by DNA extraction as described in "Materials and Methods". The result is shown in Figure 2. It shows that DNA extracted from as little as 10^2-10^3 CFU/each of both LT1h ETEC (ATCC43886) and *S. enterica* serovar Typhimurium (BCRC12947) could produce positive results. The reaction conditions for the multiplex PCR assay were optimized to ensure that all target gene sequences were satisfactorily amplified. The primers LT1L/LT1R designed in this study had the same annealing temperature as those of M1/M2; were used in each set at an almost equal annealing temperature[15], which reduced the possibility of unwanted bands originating from nonspecific amplification. Under the conditions in this study, the low sensitivity of *Salmonella* sp. might result from the competition of primers and templates. Therefore, enrichment was used to achieve successful multiplex PCR.

III. *Detection of Salmonella and LT ETEC in Samples*

In an attempt to further evaluate the specificity of

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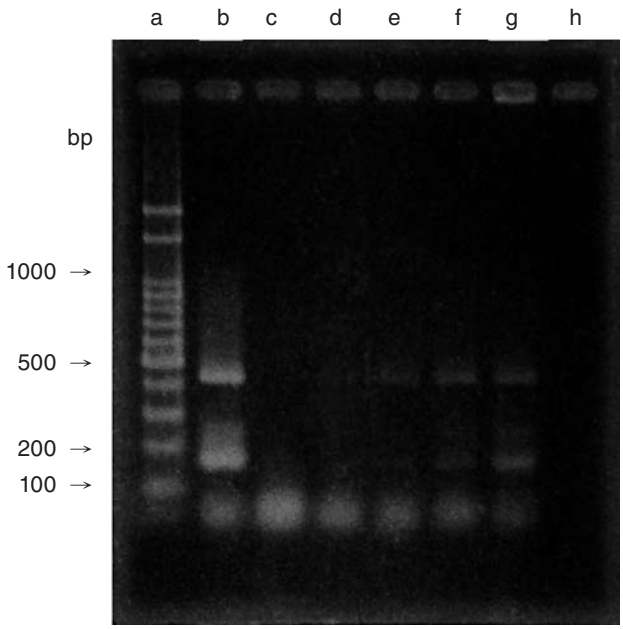

Figure 2. Sensitivity for multiplex PCR system of *S. enterica* serovar Typhimurium (BCRC12947) and LT1h ETEC (ATCC43886) using primers LT1L/LT1R and M1/M2. Lane a: 100 bp DNA ladder (Bertec Enterprise Co., Ltd. Taipei, Taiwan). Lane b: positive control. Lane c: negative control. Lane d-g: ETEC / *Salmonella* were $10^1/10^1$, $10^2/10^2$, $10^3/10^3$, $10^4/10^4$ CFU/g, respectively.

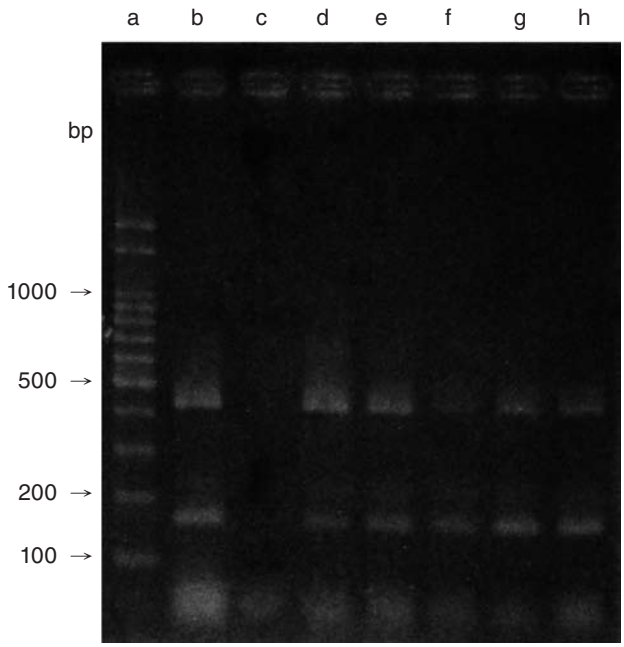

Figure 3. Detection of heat labile toxin gene of *E. coli* and of *Salmonella* strains with inoculation from 10^1-10^2 CFU target cells/g in samples after incubation with lactose broth and SCLB (selenite cystine-lactose broth). Lane a: 100 bp DNA ladder (Bertec Enterprise Co., Ltd. Taipei, Taiwan). Lane b: positive control. Lane c: negative control. Lane d: feces; Lane e: feces; Lane f: poultry; Lane g: egg; Lane h: egg.

the PCR primers and investigate the possibility of using these primers for the simultaneous detection of various *Salmonella* sp. and LT ETEC cells in foods, food samples purchased from local markets and feces were used for *Salmonella* sp. and LT ETEC detection. In general, the tested raw food samples were highly contaminated with natural microflora. For example, the viable counts in the food samples ranged from 7×10^4 to 1×10^5 CFU per gram of sample. The results showed that using inoculation ranging from 10^1 to 10^2 cells per strain per gram of food samples, *Salmonella* sp. and LT ETEC could be detected after pre-enrichment for 18 hr (Figure 3). *S. enterica* serovar Typhimurium and *E. coli* (heat-labile enterotoxigenic strains, LT ETEC) belonged to different genus and species. The problem of how to cultivate and simultaneously detect these two bacteria constitutes a major stumbling block to the detection of these bacteria by m-PCR. Inoculation of a low number of cells of *S. enterica* serovar Typhimurium and LT ETEC, mixed together in a lactose broth, followed by incubation, did not simultaneously detect *S. enterica* serovar Typhimurium *m* and *E. coli* (LT ETEC) in m-PCR. It seemed likely that growth competition between target cells and other endogenously contaminating microflora may occur.

Selenite cystine broth (SCB) is a selective medium for *Salmonella*, and Lactose broth is a growth medium for both species. A combined medium SCLB (selenite cystine broth / lactose broth, 5/3, w/w) was used to cultivate these two bacteria together and reduce the selectivity and differential effect of SCB alone. This study experimented with different ratios of SCB and lactose broth, and the ratio of 5/3 was proven the best for *Salmonella* sp. and LT ETEC detection. Figure 3 shows that after inoculation with 10^1-10^2 cells/g of *S. enterica* serovar Typhimurium and *E. coli* together into foods and followed by SCLB incubation, the presence of these two pathogens was detectable in the food. These low levels of bacterial contamination were equivalent to that frequently found in daily food. Tsen *et al.*[14] tested various cultivation methods for *Salmonella* in food to increase the sensitivity and specificity of m-PCR. Soumet *et al.*[8] used modified semisolid Rapport Vassiliadsd medium (MSRV) for subculturing *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis to enhance the sensitivity. Vantarakis *et al.*[9] analyzed *Salmonella* sp. and *Shigella* spp. in mussels and found that incubation for 24 hr in peptone water increased the detection sensitivity. Sharma and Carlson[1] reported that incubating *Salmonella* sp. and *E. coli* for 24 hr in GNTSB (prepared by mixing equal volumes of Gram-negative broth and trypticase soy broth) and using fluorogenic-PCR detection, they were able to detect 10^1-10^2 CFU/g of both bacteria in the meat products. In this study, we incubated samples inoculated with *Salmonella* and LT ETEC in Lactose broth and SCLB and obtained a sensitivity of 10^1-10^2

Table 2. Multiplex PCR for the detection of LT ETEC and *Salmonella* in samples

Sample	Analytical samples	PCR positive results by multiplex PCR		Conventional method	
		LT1L-LT1R	M1-M2	LT ETEC	*Salmonella*
Meat	35	0	1	0	1
Poultry	40	1	3	0	3
Seafood	25	0	2	0	2
Eggs	20	0	2	0	2
Feces	30	1	2	1	2
Water	10	0	0	0	0
Total	160	2	10	1	10

CFU/g; the sensitivity of m-PCR method was similar to that of Vantarakis *et al*.[9]

A total of 130 endogenous food samples from local markets and 30 fecal samples from humans were tested by conventional methods and by m-PCR methods for detection of *Salmonella* and LT ETEC as described above. One and two positive results of LT ETEC in food and fecal samples were found after enrichment for m-PCR and conventional methods (Table 2), respectively. The endogenously contaminating microflora in these samples constituted from 5×10^4 to 3×10^6 of the various samples investigated. Thus, it would seem likely that growth competition between target cells and other endogenously contaminating microflora may occur. Hence, we selected SCLB to conquer such growth-competition problems between target cells and contaminating microflora. In contrast, there was no difference in the detection rates for *Salmonella* in different methods. These results showed that m-PCR is useful and specific for the rapid detection of *Salmonella* and LT ETEC in tested samples. To assure the positive results of detection of *Salmonella* and LT ETEC in various samples containing high numbers of microflora, preculture of bacterial material was carried out prior to the PCR assay, especially using heat lysis method to prepare DNA to reduce the growth of competitive flora and rendering the less labor-intensive procedure for the *Salmonella* and LT ETEC.

In conclusion, the amplified 163 bp and 425 bp sequences in this study were unique for *Salmonella* and *E. coli*. Detection limits of the m-PCR assay for crude cell lysates of these bacteria ranged from 10^2 -10^3 CFU/each of both LT ETEC and *Salmonella*. When the protocol was applied to artificially contaminated foods and feces, results were obtained within30 hr after SCLB enrichment.

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