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A Rapid and Specific PCR Method for the Detection of *Shigella* spp. in Spiked Samples

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ABSTRACT

The purpose of this study was to develop a rapid and specific PCR system to detect *Shigella* spp in samples. A set of primers specific for the virulence gene (*Vir*F) of virulent *Shigella* spp. and enteroinvasive *Escherichia coli* (EIEC) produced specific amplicons of expected sizes of 443 bp and 331 bp by using VirF2-VirF3 and VirF3-VirF4 primer sets, respectively. These primers were then used for the detection of food with 10^1 cells/g inoculation of *Shigella* spp., followed by *Shigella* broth incubation. The presence of this pathogen in artificial contaminated foods was detected. Finally, we used this method for the detection of 100 samples, and found that none of *Shigella* spp. and EIEC bacterial strains was detected in all tested samples by PCR method and the Bacteriological Analytical Manu (BAM) method. The results indicate that the described PCR method has the advantage of a rapid, sensitivity and specificity for use in natural samples.

Key words: polymerase chain reaction, *Shigella* , enteroinvasive *Escherichia coli*

INTRODUCTION

Bacteria *Shigella* spp., belonging to the Enterobacteriaceae family, are gram-negative, facultative aerobic organisms. The virulent strains cause disease ranging from diarrhea to bacillary dysentery or shigellosis, continue to pose a threat to public health[1]. Shigellosis is spread via the fecal - oral route and is highly transmissible due to its very low infective dose (i.e. < 100 bacteria)[2]. Among *Shigella* species, *Shigella dysenteriae* has been associated with epidemic outbreaks of bacillary dysentery that pose major public health problems in developing countries and is particularly fatal to young children[3]. Enteroinvasive *Escherichia coli* (EIEC) is closely related to *Shigella* spp., there are very few characteristics that can distinguish *Shigella* strains from EIEC. Both pathogens cause dysentery and carry the *ipah* gene encoding the invasive plasmid antigen[1,2]. The *ipa*H locus is a multi-copy element present on both chromosome and invasion plasmid[3]. *Shigella* are officially divided into four groups and at least 47 serotypes, *Shigella boydii* (serovars 1-15), *S. dysenteriae* (serovars 1-10), *S. flexneri* (serovars 1-6, subserovars 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b) and *S. sonnei* (serovars 1)[4].

Human pathogens including, *Escherichia coli* O157:H7, *Salmonella* spp. and *Shigella* spp. are associated with foodborne outbreak-associated illnesses[5]. There is an urgent need for rapid, sensitive, and specific techniques for detection of these deadly bacteria. Conventional culture-based microbiological methods are laborious and time-consuming[6]. Bacterial detection methods based on nucleic acid, such as polymerase chain reaction (PCR), have shown tremendous potential and have been increasingly exploited. Investigating the applicability of the multiplex PCR in simultaneously detecting *E. coli* O157:H7 and *Shigella* present in loop-mediated isothermal amplification (LAMP)[7]. A single multiplex PCR was studied to detect diarrheagenic *Escherichia coli*[8]. Brandal *et al.*[9] studied Octaplex PCR and fluorescence-based capillary electrophoresis for identification of human diarrheagenic *E. coli* and *Shigella* spp. A combination of immunomagnetic separation (IMS) and a polymerase chain reaction (PCR) procedure was used for direct isolation and identification of *Shigella dysenteriae* type 1 and *Shigella flexneri* from feces[10,11]. Development of specific detection of *E. coli* and *Shigella* species using *uid*A[9], *ipa*H[12]and *fliC*[13] were studied. Characterization of EIEC and *Shigella* strains by randomly amplified polymorphic DNA (RAPD) analysis was also developed[14]. In order to develop a simple and less laborious method to identify EIEC as well as *Shigella* spp. in a single reaction, we have established a PCR system including only one specific virulence genes (*vir*F)[15]

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to amplify, that is different from others including Octaplex PCR[9] and multiplex PCR[8].

In this paper, we described the sensitive and specific detection of virulent *Shigella* organisms and EIEC by a single PCR reaction combined with an enrichment step. The sensitivity of the procedure was determined by using artificially seeded samples collected from natural sources.

MATERIALS AND METHODS

I. *Bacterial Strains*

The *Shigella* and *E. coli* strains used in this study included: *Shigella boydii* (BCRC15959), *Shigella dysenterias* (BCRC13983), *Shigella flexner*i (BCRC10772, 13984) and enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC) and nonpathogenic *E. coli*. A number of *Salmonella* strains, as well as strains other than *Shigella* and *E. coli,* such as *Citrobacter, Klebsiella, Enterobacter* spp. *etc.* were also used and are shown in Table 1. These bacterial strains were obtained from the American Type Culture Collection (ATCC), the Center for Disease Control (CDC), Georgia, USA; the United States Department of Agriculture (USDA), World Health Organization (WHO), Washington, USA; Department of Health (US), New York, USA; Food and Drug Administration, Taipei, Taiwan; Bioresource Collection and Research Center (BCRC), Hsin Chu, Taiwan and National Pingtung University of Science and Technology (PT), Pingtung, Taiwan.

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

II. *PCR Primers*

In this study, the PCR primer pairs designed from the *Shigella* and EIEC virulence plasmid *vir*F gene (accession number X58461) were VirF2-27(5'AGCTGCATAAGCTCT TTCTTC 3')- VirF3-469 (5'CCTCAGAATAGGAGTGTTG AA3') and VirF3-VirF4-139 (5'TCTTAGTTACTCTGTA-AACAC3') which produced DNA fragment of 443 bp and 331 bp respectively.

III. *DNA Preparation and 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 mL of the overnight culture cells were mixed with 196 μL 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 F2/F3 or F3/F4 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 s, 55°C for 50 s and 72°C for 50 s. 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 Shigella*

Overnight cultures of *Shigella dysenterias* (BCRC13983) was diluted with sterile water in decimal series. One milliliter of the diluted mixture containing *Shigella* was used for DNA extraction and PCR amplification with the procedures described above.

V. *Detection of Shigella and EIEC in Artificial Spiked Food Samples*

Meat, egg, vegetable and salad were collected from local markets. 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 - 10^3 cells/g) of *Shigella dysenterias* (BCRC13983) and *E. coli* (ATCC 43983, EIEC) were added to the homogenate. To increase the sensitivity of detection, 1 mL of the sample mixture was mixed with 9 mL of *shigella* broth (Difco, Detroit, MI, USA) and the mixture was incubated at 42°C and shaken for 16 h. All the samples were analyzed by Conventional method and PCR method as described above.

VI. *PCR Detection of Shigella and EIEC in Endogenous Samples*

Food and water were collected from local markets and feces from healthy students of Department of Food Science and Technology, Chia Nan University of Pharmacy and Science. Twenty five grams of minced sample were mixed with 225 mL of 0.1% peptone water and homogenized. To increase the sensitivity of detection, 1 mL of the sample mixture was mixed with 9 mL of *shigella* broth (Difco), and the mixture was incubated at 37°C and shaken for 16 h. All the samples were analyzed by preparing DNA for the PCR as described above, and also reconfirmed by conventional method and the PCR primers, R-F, derived from invasive plasmid antigen gene (*ipa*H)[8].

VII. *Conventional Method*

Methods as described by BAM[17] were used for *Shigella* and EIEC detection. The samples from above in *shigella* broth (Difco) were used. Such culture was plated

Table 1. Bacteria strains used and specificity of the PCR primers

Species	No. of isolates	Positive results	
		F2-F3	F3-F4
Enterobacteriaceae			
Acinetobacter calcoaceticus (ATCC 19606)	1	0	0
Alcaligenes faecalis (ATCC 8750)	1	0	0
Bacillus sutilis (ATCC 21778)	1	0	0
Brevibacterium linens (ATCC 19391)	1	0	0
Citrobacter freundii (ATCC 8090, 10787)	2	0	0
Enterobacter aerogenes (ATCC 13048, US)	2	0	0
Enterobacter cloacae (ATCC 23355)	1	0	0
Erwinia carotovora (BRCR 11298)	1	0	0
Klebsiella pneunoniae (BCRC 10692)	1	0	0
Hafnia alvei (BCRC 10906)	1	0	0
Proteus vulgaris (US)	2	0	0
Shigella			
Shigella boydii (BCRC 15959)	1	1	1
Shigella dysenterias (BCRC 13983)	1	1	1
Shigella flexneri (BCRC 10772, 13984)	2	2	2
Shigella sonnei (BCRC 10773, 10774)	2	2	2
Yersinia enterocolitica	1	0	0
Escherichia coli			
E. coli (LT & ST ETEC) (ATCC 35401, WHO 110)	2	0	0
E. coli (LT ETEC) (ATCC 37218, 33849, WH O112, 117)	4	0	0
E. coli (EIEC) (ATCC 43983, NLFD 11096, 11098)	3	3	3
E. coli (EHEC) (BCRC 13085, 13087, 13095)	3	0	0
Escherichia coli (ATCC 25922, 11775, FDB E01-E07, FDB E2416-E2422)	16	0	0
Salmonella			
Salmonella agona (PT 624)	1	0	0
S. anatum (USDA 807EI, US)	2	0	0
S. berta (US)	1	0	0
S. bonn (PT 687)	1	0	0
S. bousso (PT 643)	1	0	0
S. braenderup (PT 703)	1	0	0
S. california (US)	1	0	0
S. cerro (USDA, US)	1	0	0
S. chester (USDA)	2	0	0
S. colorado (PT 790)	1	0	0
S. coleypark (US)	1	0	0
S. cubana (USDA)	1	0	0
S. derby (CDC RF62)	1	0	0
S. djakarta (US)	1	0	0
S. emek (US)	1	0	0
S. enterica serovar Enteritidis (ATCC 13076, US)	2	0	0
S. eppendrof (PT 633)	1	0	0
S. essen (PT 661)	1	0	0
S. goerlitz (PT 645)	1	0	0
S. hadar (PT 677, US)	2	0	0
S. havana	2	0	0
S. heidelberg (CDC RF625)	1	0	0
S. hvittingfoss (USDA)	1	0	0
S. infantis (US)	1	0	0
S. Johannesburg (USDA)	1	0	0
S. kentucky (US)	1	0	0
S. kinshasa (US)	1	0	0
S. kuru (PT 793)	1	0	0
S. lagos (PT 772)	1	0	0
S. lanka (PT 660)	1	0	0
S. litchfield (PT 152, US)	2	0	0
S. london (PT 1004)	1	0	0
S. montevideo (US)	1	0	0
S. muenster (PT 1014)	1	0	0
S. ngor (PT 695-1)	1	0	0
S. nigeria (PT 696)	1	0	0
S. ohio (PT 1007)	1	0	0
S. portsmouth (PT 748)	1	0	0
S. rubislaw (US, USDA)	1	0	0
S. ruiru (USDA)	1	0	0
S. senftenberg (PT 169)	1	0	0
S. seremban (PT 1087)	1	0	0
S. Stanley (PT 639)	1	0	0
S. tananarive (PT 702)	1	0	0
S. tennessee (PT 721)	2	0	0
S. thomasville (USDA)	1	0	0
S. thompson (US)	1	0	0
S. typhi (FDB, ATCC 8427)	2	0	0
S. enterica serovar Typhimurium (ATCC 14028, e23566, BCRC 10240, PT 782)	4	0	0
S. vejle1 (PT 1102)	1	0	0
S. victoria (PT 763)	1	0	0
S. weltevreden (PT 658)	1	0	0
S. worthington (PT 705)	1	0	0

*The original sources of bacteria used in this study.

on MacConkey agar (Difco) and then incubated at 35°C for 48 h. The colony formed was transferred into triple sugar iron (TSI, Difco) agar and then incubated at 37°C for another 48 h. Further biotest and serotyping tests were performed for the identification of *Shigella* strains. For *E. coli* strains, 1 mL of the homogenous sample was inoculated into 9 mL of lauryl sulfate tryptose (LST, Difco) broth. After incubation at 35°C for 24 - 48 h, 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 *Escherichia coli* broth (EC broth, Difco) and the mixture was incubated at 45.5°C for another 24 - 48 h. Samples in gassing EC broth were streaked onto the Levine Eosin Methylene Blue agar (L-EMB, BD, Franklin Lakes, NJ, USA) plate and incubated at 35°C for another 18 - 24 h. 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.

REDULTS AND DISCUSSION

I. *Specificity of the PCR Primers*

Fragments of the DNA sequences of a virulence gene (*vir*F) of *Shigella* (accession number X58461) were selected and designed as primers for the detection of *Shigella* and EIEC. These oligonucleotide primers, termed as VirF2-VirF3 and VirF3-VirF4, were shown to be different from those analogous primers reported and prepared previously by other workers, such as the virulence plasmid *ipa*F[12] gene, a large virulence-associated plasmid pSS from *S. sonnei*[9], *uid*A[18] as well as the gene region comprising the conserved flanking regions of the 16S rRNA gene, the internal transcribed spacer region and the 23S rRNA gene (16S-ITS-23S gene region)[19].

Under the PCR conditions as described in the Materials and Methods, 7 *Shigella* spp. and 3 EIEC strains generated PCR products with molecular weight of 443 bp and 331 bp by using primer sets VirF2-VirF3 and VirF3-VirF4, respectively (Table 1, 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-EIEC or non-*Shjigella* spp., such as: *E. coli, Citrobacter* and *Salmonella* spp., etc. listed in Table 1. From the above results, the specificity of the PCR system was confirmed.

II. *Detection Sensitivity*

To test sensitivity of the assay, the PCR templates were prepared by heat lysis of the decimal serial dilution of the 20 h culture of *Shigella dysenteries* (BCRC13983). It shows that DNA extracted from as low as 10^2 CFU/mL for both primer sets VirF2-VirF3 and VirF3-VirF4 could produce positive results. The reaction conditions for the PCR assay were optimized to ensure that all target gene sequences were satisfactorily amplified. Theron *et al.*[20] identified virulent *Shigella flexneri* and enteroinvasive *E. coli* (EIEC) in spiked environment water samples by using PCR method, and a detection limit of 1.6×10^3 CFU *S. exneri* was obtained. The PCR procedure coupled with an enrichment culture incubated for 6 h detected as few as 1.6 CFU *S. flexneri*. Under

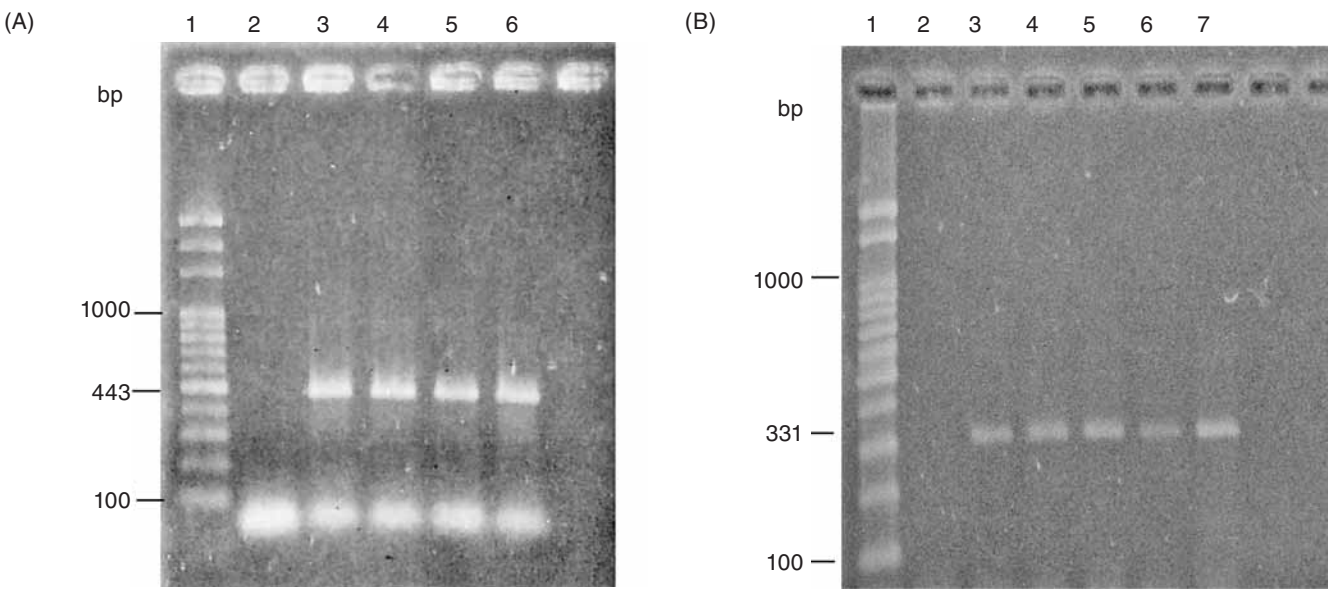

Figure 1. Specificity of polymerase chain reaction for detection of *Shigella* strains using (A) VirF2-VirF3 ; (B) VirF3-VirF4 as primers.
(A) Lane 1: DNA ladder markers; lanes 2: *E. coli* as negative control; lane 3 to 6: PCR products amplified from *Shigella boydii , S. dysenteries, S. flexneri , S. sonnei*; (B) Lane 1: DNA ladder markers; lanes 2: *E. coli* as negative control; lane 3 to 7: *S. boydii , S. dysenteries, S. flexneri , S. sonnei* and *S. flexneri.*

the conditions in this study, the low detection sensitivity of *Shigella* spp. might be conquered by enrichment to achieve successful PCR.

III. *Detection of Shigella and EIEC in Artificially Spiked Samples*

The PCR-detection method has shown that cell lysate rather than the total DNAs extracted by a phenol-chloroform method provided a sufficient quantity of bacterial DNA to allow for a rapid and simple detection procedure[21]. To assure the positive PCR detection of *Shigella dysenteries* (BCRC13983) in various samples, especially when target cells are present in very limited numbers, enrichment leading to a predominance of *Shigella dysenteries* was carried out[22]. The results showed that after inoculation with 0, 10^1 - 10^3 cells/g of *Shigella dysenterias* into samples, *Shigella dysenterias* was detected with both conventional culture method and the PCR method after pre-enrichment with *shigella* broth (Table 2 and Figure 2). Our results indicated that as few as 10^1 cells/g of the sample could constitute sufficient cellular material to generate a positive PCR results following enrichment when VirF2-VirF3 and VirF3-VirF4 were used as primers. Thus, the combination of pre-enrichment and PCR has the advantage of enhancing the sensitivity of *Shigella* and EIEC[20].

The PCR test in food samples may be limited by the presence of substances that inhibit the assay[17]. In this study, experiments with artificially challenged samples, without pre-enrichment, failed to detect *Shigella dysenterias* by PCR. Such result was also reported previously by Theron *et al.*[20]. Song *et al.*[7] studied that after 24 h enrichment, the multiplex PCR assay could concurrently detect *Shigella* and EIEC. Despite the requirement of enrichments, the method described herein was convenient and time effective because of the simple DNA preparation step. In this study, we demonstrate that the sensitivity of assay can be increased when the samples were pre-enriched.

IV. *Detection of Shigella or EIEC in Endogenous Samples*

Totally 90 endogenous food samples from local markets and 10 fecal samples from healthy humans were tested by conventional culture methods (BAM) and by PCR methods

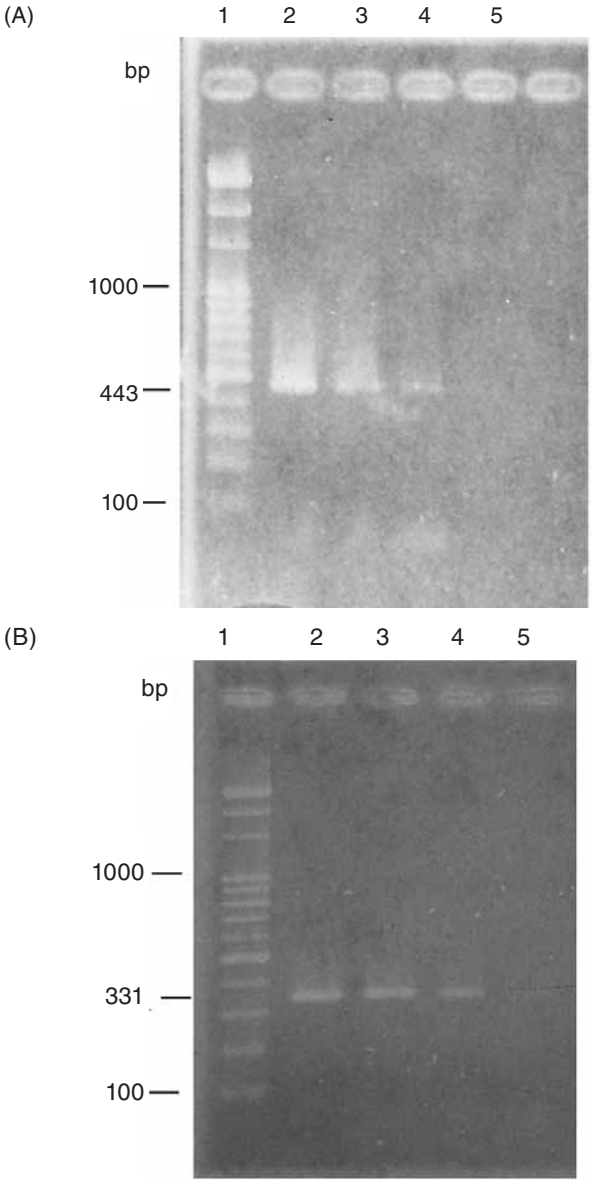

Figure 2. Detection sensitivity of polymerase chain reaction in salad samples that had been spiked with *Shigella dysenteries* and EIEC using (A) VirF2-VirF3 ; (B) VirF3-VirF4 as primers.
(A) Lane 1: DNA ladder markers. Lanes 2 - 4: PCR results amplified from 10^3- 10^1 CFU target cells g^{-1}; Lane 5: blank without inoculation of the target cells. (B) Lane 1: DNA ladder markers. Lanes 2 - 4: PCR results amplified from 10^3- 10^1 CFU target cells g^{-1}; Lane 5: blank without inoculation of the target cells.

Table 2. Detection sensitivity with and without inoculation of *Shigella dysenterias* using PCR primers

Samples	No. of samples	PCR positive results by PCR								Conventional method			
		VirF2-VirF3				VirF3-VirF4							
		0	10^1	10^2	10^3	0	10^1	10^2	10^3	0	10^1	10^2	10^3
Meat	5	0	5	5	5	0	5	5	5	0	5	5	5
Egg	10	0	10	10	10	0	10	10	10	0	10	10	10
Feces	10	0	10	10	10	0	10	10	10	0	10	10	10
Vegetable	20	0	20	20	20	0	20	20	20	0	20	20	20
Salad	10	0	10	10	10	0	10	10	10	0	10	10	10

Table 3. Detection of *Shigella* or EIEC in naturally contaminated food samples

Food	Analytic samples	PCR results		Conventional method of *Shigella* and *E. coli*	PCR results by primers R-F[a]
		VirF2-VirF3	VirF3-VirF4		
Water	30	0	0	0	0
Salad dressing	10	0	0	0	0
Vegetable	20	0	0	0	0
Salad	30	0	0	0	0
Feces	10	0	0	0	0
Total	100	0	0	0	0

a: primer pair, R-F, derived from invasive plasmid antigen gene (*ipa*H)[8].

for the detection of *Shigella* and EIEC. Table 3 shows that no amplified products were detected after pre-enrichment in food and fecal samples. Herein we found the same results as those obtained from BAM and R-F, derived from invasive plasmid antigen gene (*ipa*H)[8].

This study also indicated that shigellae without enrichment (nonculturable) failed to grow in conventional culture media. The potential health hazard presented by such *Shigella* species existing in the nonculturable state may not be able to detect such cells in the natural environment by employing routine bacteriological methods[20]. Any detection method that is employed must therefore be capable of detecting low numbers of shigellae against a large background of other cells and of organic material which may be present in the sample. The PCR test for detection of *Shigella* in faeces[23], food[24] and water samples[20] may be limited by the presence of substances that inhibit the assay[25]. However, most testing procedures describe laborious DNA extraction procedures which are necessary to eliminate substances in samples that can inhibit PCR. To minimize these problems, Theron *et al*.[20] utilized PCR technology coupled with an enrichment procedure that not only diluted PCR inhibitors but also resulted in increased numbers of *S. flexneri* organisms in reaction mixtures. In this experiment with artificially challenged samples without pre-enrichment failed to detect *Shigella* and EIEC by PCR, such result was also reported by Theron *et al*.[20].

In the present study, we examined the *Shigella* spp. and EIEC from natural samples. Direct cell lysis after enrichment may be an alternative, simple and rapid method to obtain template DNA for PCR amplification. Moreover, detection *Shigella* spp. and EIEC by the PCR method developed in this study can be completed within twenty hours as compared to the five to seven days required for bacterial culture and a conventional serological method.

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