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A Study on Detecting and Identifying Enteric Pathogens With PCR

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Objective To develop a rapid and definite diagnostic test of bacterial enteritis caused by pathogenic enterobacteria, the most frequent etiologic agent of infectious enteritis in the world. **Methods** A set of conventional PCR assays were applied to detect and identify s*almonella, shigella,* and *E. coli* O157:H7 directly from pure culture and fecal samples. The general primers of pathogenic enterobacteria were located on the uidA gene, which were found not only in *E. coli* nuclear acid, but also in *Shigella* and s*almonella* genes. *Shigella* primer was from ipaH gene whose coded invasive plasmid relative antigen existed both in plasmid and in genome. The primers of s*almonella* were designed from the 16SrRNA sequence. The primer of *E.coli* O157:H7 was taken from eaeA gene. Five random primers were selected for RAPD. The detection system included common PCR, semi-nested PCR and RAPD. **Results** This method was more sensitive, specific and efficient and its processing was rapid and simple. For example, the method could be used to specifically detect and identify s*almonella, shigella,* and *E. coli* O157:H7, and its sensitivity ranged from 3 to 50 CFU, and its detection time was 4 hours. **Conclusion** This PCR method, therefore, can serve as a routine and practical protocol for detecting and identifying pathogenic microorganisms from clinical samples.

Key words: Enteric pathogen; Detection; Identification; PCR

INTRODUCTION

Outbreak and prevalence of infective diarrhea still imperil human health[1]. *Salmonella typhi* is the pathogen of typhoid fever which is a serious public health problem in many geographic areas[2]. *Shigella* species are responsible for a substantial proportion of cases of bacillary dysentery, resulting in endemic disease and sporadic epidemics in developing countries[3]. Enterohemorrhagic *Escherichia coli* (EHEC) serotypes are associated with hemorrhagic colitis, hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura. Several EHEC serotypes have been associated with human infection[4]. All of these enterobacteria can be transmitted through water or foods. A rapid, sensitive and specific method for detection of the enteric pathogens would be helpful both in the clinical diagnosis and in epidemiological investigations of infective diarrhea during the foodborne or waterborne outbreaks. At present, few of these pathogens are detected in clinical practice, particularly in developing countries, where traditional bacteria culture and biochemical identification are usually used. These traditional methods take several days to get a result[5].

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Immunological methods and gene probe hybridization also have problems of sensitivity and specificity. PCR has been widely used to detect microorganisms by its high sensitivity and specificity and easy operation, however, only one type of bacteria can be detected with general PCR. Probe hybridization and other techniques will also be used in the process of PCR to identify pathogen compositions, but the process needs a long time and is not easy to be operated[6-8].

In order to simplify the operational process and to raise the sensitivity and specificity, universal primer PCR was used in detecting common enteric pathogens for the species and genus. At the same time semi-nested PCR and random amplification polymorphic DNA (RAPD) were used to identify the PCR products.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and DNA Extraction

The bacterial strains used in this study for developing PCR method are listed in Table 1 (All the bacterial strains were obtained from CMCC, Centers for Medical Culture Collection, Beijing, China), and for identification of the PCR are listed in Table 2 (isolated from water, foods or patients). The culture conditions for enterobacteria were described previously. The bacteria were grown in nutrient broth and maintained on nutrient agar plates. A pure culture isolate was used to inoculate broth cultures, and cells were grown to late log phase at 37℃. Total bacterial DNA was extracted as described previously[9]. Ten mL of the late log phase culture was concentrated by centrifugation at 10000 rpm for 10 min at 25℃. The supernatant was discarded and the cells were resuspended in 100 μL of distilled water, and then was lysed at 99℃ for 10 min in the Perkin-Elmer Gene-Amp™ 9600 PCR system. Following ethanol precipitation, nucleic acid preparations were dried under vacuum and resuspended in 100 μL of distilled H_2O incubated at 65℃ for 30 min and stored at 4℃ until they were used.

TABLE 1

Bacteria Used in This Study for Developing PCR Method

Strains	References
Salmonella Strains	
Salmonella typhi	CMCC 50086
Salmonella typhi	CMCC 50096
Salmonella typhi	CMCC 50098
Salmonella typhi	CMCC 50013
Salmonella typhi	CMCC 50708
Salmonella cholerae-suis	CMCC 50019
Salmonella paratyphi A	CMCC 50073
Salmonella paratyphi B	CMCC 50309
Salmonella london	CMCC 50106
Salmonella thompson	CMCC 50073
Shigella Strains	
Shigella boydii	CMCC 51582

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Strains	References
Shigella boydii	CMCC 51583
Shigella boydii	CMCC 51585
Shigella boydii	CMCC 51464
Shigella dysenteriae	CMCC 51335
Shigella dysenteriae	CMCC 51336
Shigella dysenteriae	CMCC 51570
Shigella dysenteriae	CMCC 51258
Shigella flexneri	CMCC 51207
Shigella flexneri	CMCC 51307
Shigella flexneri	CMCC 51572
Shigella flexneri	CMCC 51573
Shigella sonnei	CMCC 51334
Shigella sonnei	CMCC 480025
Escherichia coli Strains	
Escherichia coli	CMCC 8099
Escherichia coli	CMCC 285
Escherichia coli	CMCC 270015
Escherichia coli	CMCC 270016
Escherichia coli	CMCC 270017
Escherichia coli O157:H7	Hospital Isolate
EIEC	CMCC 44336
EPEC	CMCC 44338
EPEC	CMCC 44155
ETEC	CMCC 44813
Other Strain	
Vibrio arahaemolyticus	CMCC 20502
Vibrio arahaemolyticus	CMCC 20516
Protus mirabillis	CMCC 430011
Protus moganii	CMCC 430024
Enterobacter aerogenes	CMCC 41002
Enterobacter cloacae	CMCC 45301
Klebsiella ozaenae	CMCC 46110
Citrobacter	CMCC 48017
Listeria monocytogenes	CMCC 54001
Yersinia enterocolitica	CMCC 52219
Yersinia enterocolitica	CMCC 52211
Staphylococcus aureus	CMCC 26001
Legionella pneumophila	Hospital Isolate
Brucella abortus	CMCC 210101
Pseudomonas aeruginosa	CMCC 10102

PCR Amplification

The universal primers of enteric pathogen used in this study were located on the uidA gene that was coded β-glycuronidase[10]. Through detection, it was found that the fragment primer located not only in *escherichia* nucleic acid, but also in *shigella* and *salmonella* genes, whose length of the proved fragment was 480 bp[11].

The *shigella* primers were ipaH gene whose coded invasive plasmid was a relative antigen of *shigella* spp. And EIEC was present in multiple copies on both the plasmid and genome[12]. The length of the produced fragment was 422 bp.

Since there were many serotypes for *salmonella*, the primers used formerly in detecting *salmonella* had non-specificity amplification. In this study, the most conserved fragment (16SrRNA sequence) in *salmonella* genome was used as a template to design primers as well as confirmed semi-nested PCR[13], and the fragment produced was 555 bp and 437 bp respectively.

Primer design for *E.coli* was referred to the report of Meng[14]. The primers were taken from eaeA, in which the PCR product was 633 bp. Five random primers were selected for RAPD.

All the oligonucleotide primers used in the study are listed in Table 3, which were synthesized with a model 391 DNA synthesizer (PCR-Mate).

PCR assays were performed in 100-μL volumes containing 1 ng of DNA, 10 μL 10×PCR buffer, 2.5 mmol/L $MgCl_2$, 0.1 mmol/L (each) primer set, 0.2 mmol/L dNTPs, and 2.5U Taq DNA polymerase (Gibco-BRL). The reactions were carried out with a 2400 gene amplification PCR thermal cycler (PE Inc.). Thirty-five temperature cycles consisted of 3 min at 94℃, followed by 45 s at 94℃, 45 s at 55℃ and 60 s at 72℃, repeated 35 cycles. The final cycle was followed by incubation of the reaction mixture for 10 min at 72℃.

PCR products were analyzed by electrophoresis with 1.5% (wt/vol) agarose gel containing 0.5 μg of ethidium bromide per mL. These were visualized with UV illumination and photographed. DNA molecular size standards (100 bp ladder, Gibco/BRL) were included in each agarose gel electrophoresis.

RESULTS

Specificity of universal (uidA) primers in amplifying enteric pathogens Several types of experimental bacteria (49 strains) were selected to test the specificity of the uidA primer. As can be seen from Table 4, *salmonella, shigella* and *E. coli* species were positive, and no result was found in other strains. The results showed that this pair of primers was highly specific.

Results of shigella (ipaH) primer amplifying With specificity test, it was shown that besides every *shigella* strain, a 442 bp fragment was found in enteroinvasive *E.coli* (EIEC) 44336, other bacteria were negative (Table 4). The sensitivity of this pair of PCR primers was tested by a serial dilution of the pure *shigella* strains. The lowest detection level was 50 cfu per reaction system (Fig. 1).

Identification of specificity by semi-nested PCR The specificity was also confirmed by nested-PCR with the conditions determined by the first PCR, and the products of the first experiment were used as the templates of semi-nested PCR. With the semi-PCR primers (ipaH1 and ipaH3), an expected 307 bp was obtained only with *shigella* strains and EIEC. The detection limit for *Shigella sonnei* 51334 after 35 cycles of reaction was increased to 5 cfu (Fig. 2).

TABLE 2

Bacteria for Identification of the PCR

No.	Hybridization Assay	Resources
1	*Salmonella* spp.	Food
2	*Salmonella* spp.	Food
3	*Salmonella* spp.	Food
4	*Salmonella* spp.	Food
5	*Salmonella* spp.	Food
6	*Salmonella* spp.	Food
7	*Salmonella* spp.	Water
8	*Shigella* spp.	Water
9	*Shigella* spp.	Food
10	*Shigella* spp.	Food
11	*Shigella* spp.	Food
12	*Shigella* spp.	Patient
13	*Shigella* spp.	Patient
14	*Shigella* spp.	Patient
15	*EPEC*	Patient
16	*E. coli* O157:H7	Food
17	*E. coli* O157:H7	Food
18	*E. coli* O157:H7	Water
19	*Vibrio parahaemolyticus*	Water
20	*Vibrio parahaemolyticus*	Food
21	*Vibrio parahaemolyticus*	Food
22	*Legionella pneumophila*	Water
23	*Legionella pneumophila*	Water
24	*Legionella pneumophila*	Water
25	*Legionella pneumophila*	Water
26	*Legionella pneumophila*	Water

TABLE 3

Oligonucleotide Primers Used in the Study

Primers		Oligonucleotide Sequence (5′—3′)	Location Within Gene	PCR Product Size (bp)
uidA	1	TTCGCCGATGCAGATATTCG	1628	
	2	AACGCTGACATCACCATTGG	2107	480
ipaH	1	TGGAAAAACTCAGTGCCTCT	1149	
	2	CCAGTCCGTAAATTCATTCT	1570	422
	3	AGCTGAAGTTTCTCTGCGAGC	1455	307[a]
16Ss	1	TGTTGTGGTTAATAACCGCA	454	
	2	CACAAATCCATCTCTGGA	1008	555
	3	CCGTCAATTCATTTGAGTTT	926	473[a]
eaeA	1	CCATAATCATTTTATTTAGAGGGA	1	
	2	GAGAAATAAATTATATTAATAGATCGGA	633	633
rapd[b]	1	TGATCCCTGG		
	2	AGCCTGAGCC		
	3	GGTGCTCCGT		
	4	CGGAGGCGTC		
	5	ACGTGGACGG		
	6	CCAGACCCTG		

Note. [a]semi-nested PCR primer. [b] RAPD primer.

Detection of salmonella with PCR All *salmonella* species can be detected with the 16Ss primers, and the product was 555 bp (Table 4). The sensitivity was tested with s*almonella* 50013 as a representative. The bacteria could also be detected when it was as low as 30 cfu.

Identification with semi-nested PCR The semi-PCR with 16Ss1 and 16Ss3 could get the expected product of a 473 bp fragment. The sensitivity of this pair of PCR primers in detection of pure s*almonella* 50013 genomic DNA after 35 cycles' reaction was 3 cfu.

Detection of E. coli O157:H7 It was shown that *E. coli* O157:H7 and *salmonella cholera suis* strain yielded a positive result with the eaeA primers. The fragment length was 633bp that was the same as the length designed (Table 4).

Differentiate O157:H7 *and Salmonella cholera suis with RAPD technique* Five random primers (S571, S397, S440, S528 and S756) were used in RAPD test to differentiate O157:H7 and *salmonella cholera suis*. It was found that only the S 571 primer could amplify and differentiate these bacterial DNA simultaneously through the electrophoregram.

Conditions of RAPD fingerprinting PCR was carried out with the annealing temperature of 20℃, 25℃, 28℃, 35℃ and 45℃. It was showed that many bands would appear and the result would not be stable if the annealing temperature was lower than 28℃. However, the bands would be decreased significantly or give the negative result when the annealing temperature was higher than 28℃. The optimal annealing temperature for RAPD was 28℃.

TABLE 4

Bacterial Strains and PCR Results

Strains		uidA	ipaH	16Ss	eaeA
Salmonella Strains					
Salmonella typhi	CMCC 50086	+	-	+	-
Salmonella typhi	CMCC 50096	+	-	+	-
Salmonella typhi	CMCC 50098	+	-	+	-
Salmonella typhi	CMCC 50013	+	-	+	-
Salmonella typhi	CMCC 50708	+	-	+	-
Salmonella cholerae-suis	CMCC 50019	+	-	+	+
Salmonella paratyphi A	CMCC 50073	+	-	+	-
Salmonella paratyphi B	CMCC 50309	+	-	+	-
Salmonella london	CMCC 50106	+	-	+	-
Salmonella thompson	CMCC 50073	+	-	+	-
Shigella Strains					
Shigella boydii	CMCC 51582	+	+	-	-
Shigella boydii	CMCC 51583	+	+	-	-
Shigella boydii	CMCC 51585	+	+	-	-
Shigella boydii	CMCC 51464	+	+	-	-
Shigella dysenteriae	CMCC 51335	+	+	-	-
Shigella dysenteriae	CMCC 51336	+	+	-	-
Shigella dysenteriae	CMCC 51570	+	+	-	-
Shigella dysenteriae	CMCC 51258	+	+	-	-
Shigella flexneri	CMCC 51207	+	+	-	-
Shigella flexneri	CMCC 51307	+	+	-	-
Shigella flexneri	CMCC 51572	+	+	-	-
Shigella flexneri	CMCC 51573	+	+	-	-
Shigella sonnei	CMCC 51334	+	+	-	-

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Strains		uidA	ipaH	16Ss	eaeA
Shigella sonnei	CMCC 480025	+	+	-	-
Escherichia coli Strains					
Escherichia coli	CMCC 8099	+	-	-	-
Escherichia coli	CMCC285	+	-	-	-
Escherichia coli	CMCC 270015	+	-	-	-
Escherichia coli	CMCC 270016	+	-	-	-
Escherichia coli	CMCC 270017	+	-	-	-
Escherichia coli O157:H7		+	-	-	+
EIEC	CMCC 44336	+	+	-	-
EPEC	CMCC 44338	+	-	-	-
EPEC	CMCC 44155	+	-	-	-
ETEC	CMCC 44813	+	-	-	-
Other Strain					
Vibrio arahaemolyticus	CMCC 20502	-	-	-	-
Vibrio arahaemolyticus	CMCC 20516	-	-	-	-
Protus mirabillis	CMCC 430011	-	-	-	-
Protus moganii	CMCC 430024	-	-	-	-
Enterobacter aerogenes	CMCC 41002	-	-	-	-
Enterobacter cloacae	CMCC 45301	-	-	-	-
Klebsiella ozaenae	CMCC 46110	-	-	-	-
Citrobacter	CMCC 48017	-	-	-	-
Listeria monocytogenes	CMCC 54001	-	-	-	-
Yersinia enterocolitica	CMCC 52219	-	-	-	-
Yersinia enterocolitica	CMCC 52211	-	-	-	-
Staphylococcus aureus	CMCC 26001	-	-	-	-
Legionella pneumophila	Hosp. isolate	-	-	-	-
Brucella abortus	CMCC 210101	-	-	-	-
Pseudomonas aeruginosa	CMCC 10102	-	-	-	-

Identification of E. coli and salmonella cholera suis with RAPD RAPD was carried out with S571 primer by using DNAs extracted from *E. coli* and *salmonella cholera suis.* Representative results are shown in Fig. 3. It was revealed that the RAPD profile of O157:H7 was 4 bands and that of *Salmonella cholera suis* was 2 bands on the agarose gel. *E. coli* O157:H7 could be easily distinguished form *Salmonella cholera suis* by electrophoresis in bands of the amplified products.

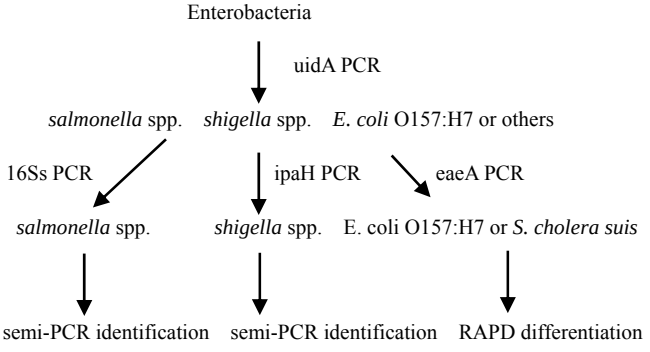
Detection of practical samples We collected and analyzed a series of 26 bacterial strains from patients, food or water which were confirmed by special culture and conventional methods. The following organisms were isolated: *Salmonella* species (7), *Shigella* species (7), *E.coli* O157:H7 (3) and other bacteria (9). While uidA –PCR was positive in the 18 bacterial strains, of them 6 strains were positive using 16Ss primers, 7 strains positive using ipaH primers and 3 strains positive with eaeA primers PCR in combination with RAPD. However, one EIEC was identified as *salmonella* species by PCR (Table 5).

TABLE 5

Comparison of Detection Results for Bacteria With PCR and Conventional Methods

No.	Hybridization Assay	Conventional Methods	Consistency
1	*Salmonella* spp.	*Salmonella* spp.	Y[a]
2	*Salmonella* spp.	*Salmonella* spp.	Y
3	*Salmonella* spp.	*Salmonella* spp.	Y
4	*Salmonella* spp.	*Salmonella* spp.	Y
5	*Salmonella* spp.	*Salmonella* spp.	Y
6	*Salmonella* spp.	*Salmonella* spp.	Y
7	*Salmonella* spp.	EIEC	Nb
8	*Shigella* spp.	*Shigella* spp.	Y
9	*Shigella* spp.	*Shigella* spp.	Y
10	*Shigella* spp.	*Shigella* spp.	Y
11	*Shigella* spp.	*Shigella* spp.	Y
12	*Shigella* spp.	*Shigella* spp.	Y
13	*Shigella* spp.	*Shigella* spp.	Y
14	*Shigella* spp.	*Shigella* spp.	Y
15	*EPEC*		—c
16	*E. coli* O157:H7	E. coli O157:H7	Y
17	*E. coli* O157:H7	E. coli O157:H7	Y
18	*E. coli* O157:H7	E. coli O157:H7	Y
19	*Vibrio parahaemolyticus*		—
20	*Vibrio parahaemolyticus*		—
21	*Yersinia enterocolitica*		—
22	*Legionella pneumophila*		—
23	*Legionella pneumophila*		—
24	*Legionella pneumophila*		—
25	*Legionella pneumophila*		—
26	*Legionella pneumophila*		—

Note.[a]The two results, from both the PCR and the conventional methods, were consistent. [b]The two results were inconsistent. [c]Could not be detected.



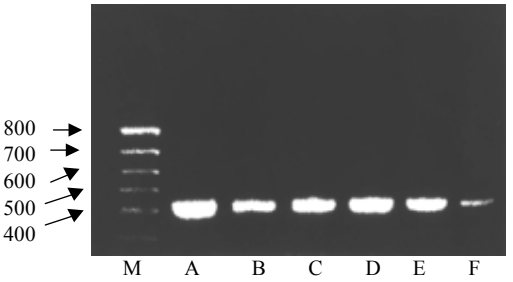


FIG. 1. Specific amplification of ipaH locus in enterobacteria. Lane M: DNA ladder, Lane A-F:51334, 51570, 51573, 51582, 51585, 44338.

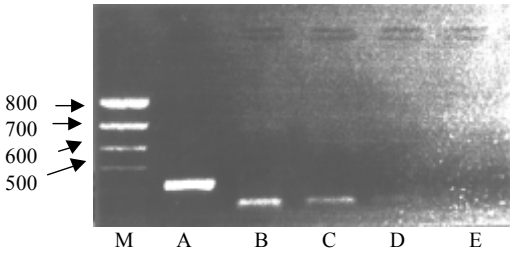


FIG. 2. Sensitivity of the semi-PCR. Lane M: DNA ladder, Lane A: product of PCR, Lane B-E: products of semi-PCR with 5×101, 5×100, 5×10^{-1}, and 0 cfu.

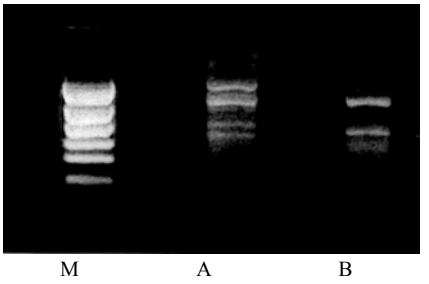


FIG. 3. Identification of RAPD bands in *E.coli* O157:H7 and Sal.cholerae-suis. Lane M: DNA ladder, Lane A: RAPD products of E.coli O157:H7, Lane B: RAPD products of Sal.cholerae-suis.

DISCUSSION

Salmonella spp., *shigella* spp. and *E.coli* O157:H7 are the most important enteric pathogens in water and foods, which can cause acute dysenteric syndrome[1]. It is time consuming to detect enteric bacteria from clinical samples with traditional method. Besides, the detectable rate is markedly affected by the presence of other bacteria competitive

inhibitions in samples. Or bacteria can not be cultured because of interference with materials. Gene probe hybridization can raise the specificity and sensitivity, but it is complicated to operate and needs a long time.

In detecting bacterial genetic material (DNA), PCR can overcome the shortcomings of the above methods. Up to now, PCR has been widely used in microbiology. We analyzed uidA gene which is a coded β-glycuronidase in *E. coli*, and found that there were some homologous sequences between uidA gene and *shigella* or *salmonella* genomes[10,11]. So primers uidA1 and uidA2 were designed based on this gene fragment. After improving the reaction conditions, a similar band was only produced in *E. coli, salmonella* and *shigella*, but all the others were negative. Therefore, the universal primer PCR is useful to identify whether the three common pathogens exist or not.

It was reported that the primers from invA gene sequence were used to detect *salmonella*, but they gave some false negative results[15]. Primers from IS200 insertion sequence and HimA gene could not avoid negative results since not all *salmonella* contained these sequences[16]. The ribosomal RNA gene is genetically stable and consists of conserved and variable regions. The variable region may vary considerably among different bacterial species and can be therefore used as a target for PCR detection. In this study the 16s rRNA sequence was chosen as a template, on which a pair of primers was designed. After improving the reaction conditions, only *salmonella* could be amplified specifically, but others were negative. The sensitivity was 30 cfu. In addition, a piece of semi-nested primer between these two primers was designed to identify the amplified products. The detecting sensitivity was raised to 3 cfu.

Besides four kinds of *Bacillus dysenteria*, pathogens of dysentery also contain invasive *E.coli*. It has been proved that this bacterium and all *Bacillus dysentery* carry invasive unconjugated large plasmid, which codes several kinds of invasion related envelop membrane protein. Formerly, false results were often present in tests when a pair of primers was designed in a piece of invasion related site on the plasmid, since this area had a higher natural deletion mutation ratio[17]. Although the ial gene cluster was specific for the shigellae and EIEC invasive phenotypes, spontaneous loss of the invasive plasmid or selective deletion of the invasion-associated plasmid-encoded genes might limit the usefulness of the ial[6]. The ipaH gene is located on a 4.6-kb *Hind*Ⅲ fragment that is derived from a 39-kb segment of the invasion plasmid, which was found to restore invasion to a plasmid-cured *shigella* strain and to code for several outer membrane proteins of the bacterium, termed invasion plasmid antigens (ipa), that were immunogenic in *shigella*-infected humans[6]. When used as a DNA probe, this region was shown by Venkatesan[17] to hybridize specifically with all tested virulent *shigella* and EIEC strains. The ipaH gene was found to multicopy, and chromosome genome contained homologous sequence[18]. Therefore, the ipaH gene was selected as a template to design the primers ipaH1 and ipaH2 in order to avoid false negative results. The semi-nested PCR primers (combined with ipaH3 and ipaH1) were designed between this pair of primers. A 422 bp fragment was produced in all *shigella* and EIEC when annealing temperature was at 50℃-60℃, while other bacteria were negative. Semi-nested PCR not only could be used to identify the correctness of the first PCR product, but also could improve the sensitivity. It is clear that the ipaH primer is more sensitive and specific than the ia1 primer in detecting *shigella* spp. and EIEC.

E.coli contains both pathogenic strains (such as EIEC, enterotoxingenic *E. coli*, enteropathogenic *E. coli*, enterohemorrhagic *E. coli* and so on) and non-pathogenic strains. We composed a pair of primers using eaeA gene to detect *E. coli* O157:H7. The eaeA gene encodes the protein intimin, which is thought to be responsible for the close association of EHEC and EPEC isolates to the cytoplasmic membrane of cell lines such as Hep-2 *in vitro*

and enterocytes *in vivo*. The 5′portion of the eaeA gene appears to be relatively well conserved among EHEC and EPEC isolates[7,14,19]. In this studies, the eaeA primers were shown to amplify DNAs from *E. coli* O157:H7 and *salmonella cholera suis* but not DNAs from other enterobacteria. RAPD technique was used to differentiate *E. coli* O157:H7 from *Salmonella cholera suis,* which was simple, easy and specific.

In summary, a systematic and rapid method for detection and identification of enteric pathogens was developed in this study, and the detail procedures were illustrated as follows:

The greatest advantage of the above PCR detection method for enteric pathogens is its speed of detection. Only four hours are required from manipulating samples to reading results. This is a breakthrough over the traditional detection method, which requires more than 48 h. Other advantages include its high sensitivity, specificity and easy operation. Furthermore, no radio-isotopes and their concomitant dangers are involved.

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