

Genetic detection of diarrheagenic *Escherichia coli* isolated from children with sporadic diarrhea

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Escherichia coli strains are among the major bacterial causes of diarrheal illness. At least 5 categories of diarrheagenic *E. coli* (DEC) are recognized, namely enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and enterohemorrhagic *E. coli* (EHEC). Due to the need for costly and labor-intensive diagnostic procedures, identification of DEC is difficult at standard laboratories. Therefore, the epidemiology of DEC infections remains obscure in Taiwan. Recently, polymerase chain reaction (PCR) or dot blot has been used for genetic detection of DEC. In this study, we analyzed 150 *E. coli* isolates from diarrheal stools of children under 5 years old. The PCR tests detected 5 ETEC (3.3%), 6 EPEC (4%), 4 EIEC (2.7%), and 13 EAEC (8.7%) isolates. No EHEC was detected. Dot blot and sequence analysis were used to confirm the results of PCR. The cellular fatty acid (CFA) profiles from *E. coli* isolates were also analyzed. Comparison of CFA composition revealed minor variation in the percentage of each fatty acid detected among DEC isolates of ETEC, EPEC, EIEC and EAEC, but did not provide enough evidence for differentiating between categories of DEC by CFA profiles alone.

Key words: Diarrhea, *Escherichia coli*, PCR, preschool child

Infection with diarrheagenic *Escherichia coli* (DEC) has been an increasing clinical concern. The DEC strains include: 1) enterotoxigenic *E. coli* (ETEC) strains, which are associated with traveler's diarrhea; 2) enteropathogenic *E. coli* (EPEC) strains, which cause diarrhea in children; 3) enteroinvasive *E. coli* (EIEC) strains, which are involved in invasive intestinal infections, watery diarrhea and dysentery; 4) enteroaggregative *E. coli* (EAEC) strains, which are associated with persistent diarrhea; and 5) enterohemorrhagic *E. coli* (EHEC) strains, which are associated with hemorrhagic colitis and hemolytic-uremic syndrome [1-3].

Although some serogroups were predominant in specific categories of DEC, these markers are rarely sufficient in and of themselves to reliably identify a strain as diarrheagenic [4]. With the exception of EHEC, O157:H7, no biochemical or serological markers can

be used definitely to differentiate DEC from normal inhabitant strains [5]. Traditionally, the identification of this kind of pathogen is based on its phenotype. Detection of ETEC usually depends on demonstration of toxin production by specific bioassays or enzyme-linked immunosorbent assay. ETEC produces heat-labile enterotoxins (LT) or heat-stable enterotoxins (ST) [1-4,6]. The phenotypic identification of EPEC requires the use of cell cultures and fluorescence microscopy [1,6]. The classical phenotypic assay for EIEC is the Sereny test. EAEC strains are characterized by their unique patterns of adherence to HEp-2 cells. The bacteria appear as "stacked brick" clumps which adhere to both the HEp-2 cells and the glass coverslip [1]. To obtain a better understanding of the epidemiologic patterns of local pathogenic *E. coli*, however, more rapid and efficient methods for detection are needed. Molecular biology techniques for characterizing clinical isolates are thus becoming increasingly important.

This study applied polymerase chain reaction (PCR) techniques, DNA hybridization, and gas chromatography in the characterization of different

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Table 1. Characterization of 4 reference strains obtained from the CDC

CDC reference strains	Characterization	Serotype
72-5474	LT-and ST-producing	O15:H11
B2872	LT-producing	O88
TX-1	ST-producing	O78:H12
TD-213	Invasive gene	NA

Abbreviations: CDC = Centers for Disease Control, USA; LT = heat-labile enterotoxins; ST = heat-stable enterotoxins; NA = not available

subgroups of pathogenic *E. coli* isolated from fecal samples of children with diarrhea.

The MIDI Microbial Identification System (Microbial ID Inc., Newark, DE, USA) enables diagnostic laboratories to perform cellular fatty acid (CFA) analysis on a wide variety of bacteria. This system comprises a gas chromatography unit with a flame ionization detector along with Hewlett-Packard 7673A autosampler, an integrator and a computer. It can differentiate more than 140 compounds including fatty acid methyl esters (FAME), dimethyl acetyls (DMA), aldehydes (ALDE) and unknown compounds that are distinctive for individual species. This study also examined the value of rapid identification of *E. coli* by CFA analysis as a tool for differentiation between diarrheogenic and non-pathogenic strains of *E. coli*.

Table 2. Polymerase chain reaction primers used in this study targeting various virulence genes of diarrheogenic *Escherichia coli*

Category	Target gene	Primer sequence (5' to 3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
ETEC	LT	GCG ACA AAT TAT ACC GTG CT CCGAATTCTGTTATATATGT	50	708	7
	ST	CACCCGGTACARGCAGGATT ATTTTTMTTCTGTATRTCTT	50	190	8
EPEC	BFP	CAATGGTGCTTGCGCTTGCT GCCGCTTATCC AACCTGGT	55	324	7
	EAF	CAG GGT AAA AGA AAG ATGATA A TAT GGG GAC CAT GTA TTA TCA	60	397	9
EIEC	lpaH	GCT GGA AAA ACTCAGTGCCT CCAGTCCGTAATTCATTCT	55	424	10
EAEC	pCVD432	CTGGCG AAA GAC TGT ATC AT CAATGT ATAGAA ATCCGCTGTT	53	630	11
	<i>aggR</i>	CTA ATTGTACAATCGATGTA ATGA AGTAATTCTTGAAT	55	308	12
EHEC	Stx1	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	60	180	13
	Stx2	GGCACTGTCTGAACTGCTCC TCGCCAGTTATCTGACATTCTG	60	255	13

Abbreviations: ETEC = enterotoxigenic *E. coli*; EPEC = enteropathogenic *E. coli*; EIEC = enteroinvasive *E. coli*; EAEC = enteroaggregative *E. coli*; EHEC = enterohemorrhagic *E. coli*; LT = heat-labile enterotoxins; ST = heat-stable enterotoxins; BFP = bundle-forming pili; EAF = EPEC adherence factor; abbreviations in primer sequence: M = A + C, R = A + G

Materials and Methods

Bacterial strains

150 strains of *E. coli* isolates from children (aged under 5 years) with sporadic diarrhea treated at National Taiwan University Hospital during 1999 and 2000 were studied. Cultures for *Salmonella*, *Shigella*, and *Campylobacter* were all negative for these patients. Four reference strains obtained from the Centers for Disease Control and Prevention of the USA (CDC) were used as controls for the following tests (Table 1).

PCR and sequencing

DNA was prepared from cultures with a DNA isolation kit (Puregene, Genra Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. Detection of 5 categories of DEC was performed by PCR using 9 sets of primers targeting various virulence genes, as listed in Table 2 [7-13]. The PCR conditions used were as described in the references for the primers. The amplification products were subsequently subjected to gel electrophoresis (FMC BioProducts, Rockland, Maine, USA), stained with ethidium bromide and photographed under ultraviolet (UV) light.

Amplification fragments were sequenced on an Applied Biosystem Model ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA)

using the Taq BigDye-Deoxy Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions.

Dot blot assays

The dot blot assay was also used as an alternative method to verify the specificity of the above PCR assay. The PCR product was labeled with digoxigenin and then used as probe for hybridization to genomic DNAs of organisms. Probes were produced using the PCR method described above and simultaneously labeled by incorporation of digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany). For each strain tested, 300 ng of chromosomal DNA was denatured by heating at 96°C for 10 min and spotted onto Hybond-N nylon membranes (Amersham Pharmacia Biotech, UK). DNA was then fixed on to the filter by UV treatment at an intensity of 120 mJ/cm² for 3 min on a UV cross-linker. The prehybridization and hybridization temperature were both 61°C. All filters were pre-hybridized for 1 h in 5 × SSC (1.5 M sodium chloride, 0.15 M sodium citrate). Hybridization was carried out overnight with heat-denatured probe. Detection was performed using an antidigoxigenin antibody conjugated to alkaline phosphatase as a substrate according to the manufacturer's instructions.

Cellular fatty acid analysis

Bacterial strains for CFA analysis were grown on 5% sheep blood agar at 37°C under aerobic conditions for 24 hours. The CFA were extracted and derived to methyl esters according to previously described procedures [7]. Briefly, the cells were pelleted, saponified with NaOH heated in a boiling water bath for 30 min, methylated with methanol-HCl heated at 80°C for 10 min, and extracted with hexane and methyl tert-butyl ether. The organic layer was extracted with diluted NaOH. The washed organic extract was placed in an autosampler vial with an aluminum crimp cap. Specimens were processed on a Hewlett-Packard 5890A gas chromatograph (capillary column) in accordance with the manufacturer's specifications. Peak naming and column performance was achieved using calibration samples. An external calibration mixture was analyzed on each day of testing, and was automatically rerun and evaluated after every tenth sample. A software library of fatty acid compositions was searched and the fatty acid profile of the isolate was compared with those of known species, and a report was generated. The software library, CLIN40, version 4.0 of MIDI

Microbial Identification System was used to identify the strains.

Results

In total, 150 *E. coli* strains isolated from children with sporadic diarrhea were evaluated by PCR to detect 9 virulence genes to enable classification according to the DEC categories. ETEC, EPEC, EIEC, EAEC and EHEC were defined as strains having the respective virulence genes. PCR primers amplified fragments of DNA of the predicted size from CDC reference strains which were used as positive controls. The diarrheagenic strains detected by PCR included 5 ETEC, 6 EPEC, 4 EIEC and 13 EAEC. No EHEC was detected. The results of gel electrophoresis of amplification products from example isolates are shown in Fig. 1. Amplification products of EAEC with pCVD432 primers were further digested with *AluI* and *DraI*, respectively (Fig. 1E). Although the product from lane 2 was weak and had another nonspecific band, the restriction patterns, dot blot, and sequence data from this isolate all revealed a positive result for EAEC (data not shown).

The 5 ETEC isolates were detected by either the presence of genes encoding LT (4 isolates) or ST (1 isolate). Six EPEC isolates were detected by PCR amplification using primers derived from the bundle-forming pili (BFP) gene and EPEC adherence factor (EAF) sequences of EPEC. Four EIEC isolates were detected by PCR using primers targeting the *IpaH* genes. A total of 13 EAEC isolates were detected by PCR using primers targeting the fragment of pCVD43 or *aggR* genes.

The specificity of the above PCR assays was further examined by sequence analysis and/or dot blot assay. Sequencing from all of the PCR products with expected size revealed the correct amplification. Two of the sequences of amplicons obtained by using the primers targeting pCVD43 from EAEC isolates are shown in Fig. 2. The sequences of these isolates revealed the highest similarity with the published sequence of the EAEC pCVD43 fragment. By dot blot assay, only chromosomal DNA from PCR-positive strains showed a strong hybridization signal. No hybridization signal was detected by dot blot analysis of DNAs from non-pathogenic *E. coli*. The dot blot results of the ETEC strains are shown in Fig. 3.

The major amounts of CFA derivatives detected in DEC isolates were hexadecanoate (16:0), cis-9, 10-methylenehexadecanoate (17:0, cyclo), followed by hexadecenoate (16:1), octadecenoate (18:1) or cis-9,

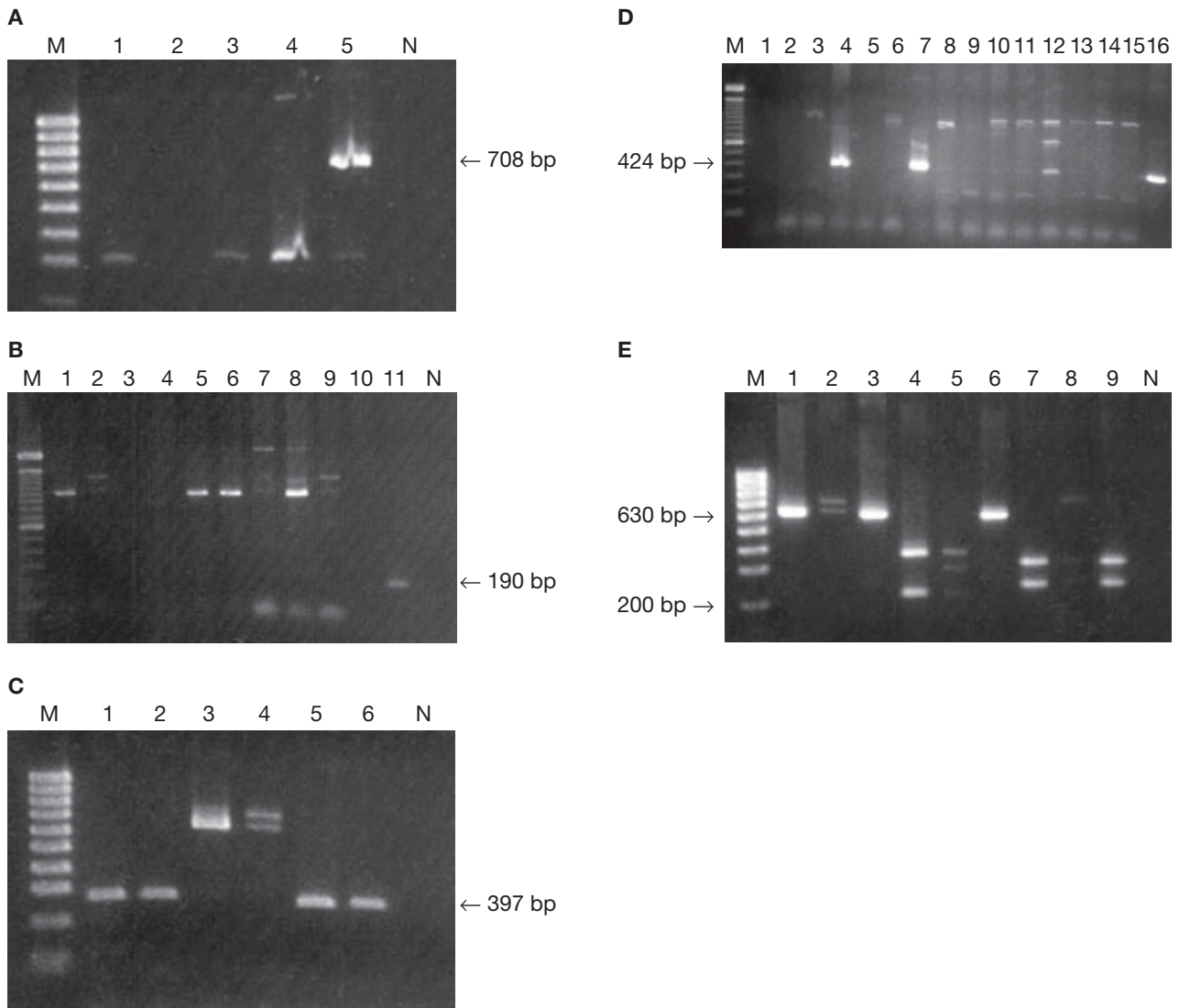


Fig. 1. Agarose gel electrophoresis of polymerase chain reaction (PCR)-amplified products from clinical isolates of *E. coli*. (A) ETEC (LT, amplification product 708 bp). Lane 5 is positive. (B) ETEC (ST, 190 bp). Lane 11 is positive. (C) EPEC (EAF, 397 bp). Lanes 1, 2, 5 and 6 are positive. (D) EIEC (424 bp). Lanes 4, 7, 12 and 16 are positive. (E) EAEC (630 bp). Lanes 1, 2 and 3 (NTUH155) are positive. Lanes 4 to 6, *AluI* digestion fragments from PCR products of lanes 1, 2 and 3, respectively; lanes 7 to 9, *DraI* digestion fragments from PCR products of lanes 1, 2 and 3, respectively. Lane M, DNA size marker (100-bp ladder). Lane N, negative control. ETEC = enterotoxigenic *E. coli*; EPEC = enteropathogenic *E. coli*; EIEC = enteroinvasive *E. coli*; EAEC = enteroaggregative *E. coli*; LT = heat-labile enterotoxins; ST = heat-stable enterotoxins; EAF = EPEC adherence factor; NTUH = National Taiwan University Hospital; PCR = polymerase chain reaction.

10-methyleneoctadecanoate (19:0 cyclo w8c). Minor amounts of other fatty acids were also detected (Table 3). All of the *E. coli* isolates could be identified to the species level using MIDI Microbial Identification System software (Library CLIN40, version 4.0). Further comparison of CFA composition revealed minor variation in the percentage of each fatty acid among the DEC isolates of ETEC, EPEC, EIEC and EAEC (Table 3), which was insufficient for differentiation among diarrheagenic categories.

Discussion

Diarrhea caused by DEC remains an important source of morbidity and mortality worldwide [14]. The epidemiological significance of each *E. coli* category in childhood diarrhea may vary with the geographical area. Only limited data on DEC have been reported from Taiwan [15]. This study examined the incidences of diarrheal illnesses of children caused by the different categories of DEC. Due to the difficulty of performing

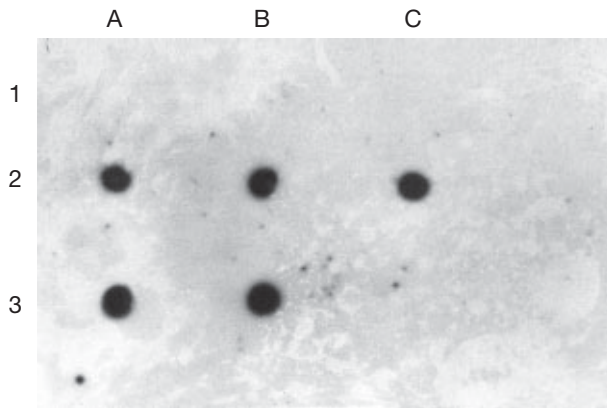


Fig. 3. Dot blot of 9 enterotoxigenic *Escherichia coli* isolates. The digoxigenin-labeled polymerase chain reaction product of heat-labile enterotoxins gene was used as probe. Chromosomal DNA from *E. coli* isolates were used as targets. There were 2 positive controls, CDC-72-5474 (dot A3) and CDC-B2872 (dot B3). Dots A1, B1, C1 and C3 are negative. Dots A2, B2 and C2 are positive.

phenotypic assays of DEC in our laboratory, a PCR-based diagnostic assay was used for the classification of *E. coli* isolates associated with diarrhea. We chose 1 or 2 sets of PCR primers to identify each DEC category. Among the 150 *E. coli* isolates, 5 ETEC, 6 EPEC, 4 EIEC and 13 EAEC were detected by PCR assay.

In this study, EAEC was found in 8.7% (13 of 150) of cases and appeared to be the most prevalent pathogen among DEC categories. EAEC appears to have recently emerged as a pathogen causing diarrhea in both developing and industrialized countries [16-18]. Sarantuya et al recently reported that the incidence of EAEC was 15.1% in Mongolian children, and was

higher in the diarrheal group than in any other category (0 to 6.0%) [17]. Previous reports showed that EAEC strains may be heterogeneous, and no DNA sequence was demonstrated to be present in all strains [16-18]. Among the virulence genes of EAEC which have been studied, *aggR* was found most frequently in strains of EAEC [17,18]. To increase the sensitivity, we used 2 genetic targets, pCVD432 and *aggR* (transcriptional activator of AAF/I and AAF/II) for identification of EAEC. Although PCR detection is rapid, the HEp-2 cell adherence assay is still the most accurate method of defining this category.

EPEC, an important pediatric diarrheal pathogen, employs multiple adhesins to colonize the small bowel and produces characteristic “attaching and effacing” (A/E) lesions on small intestinal enterocytes. EPEC adhesins that have been associated with A/E adhesion and intestinal colonization include BFP, EspA filaments and intimin. BFP are involved in bacteria-bacteria interaction and microcolony formation [19]. To increase the sensitivity and specificity, 2 sets of PCR primers were used to detect EPEC in this study. One detects *bfp* gene and the other detects EAF plasmid. BFP is encoded on EAF plasmids, which share extensive homology among various EPEC strains. The importance of the EAF plasmid in human disease was shown by Levine [3]. The EAF plasmid has been reported to control the expression of the *eeA* gene product, intimin [2]. The *eeA* gene has also been used as a target for the detection of EPEC [20]. The prevalence of EPEC may be decreasing. A report showed that, although previously common in Brazil, EPEC strains of serogroups O55,

Table 3. Comparison of cellular fatty acid composition of diarrheagenic *Escherichia coli* isolates

Cellular fatty acid	Percent (mean) of total chromatographic area			
	Diarrheagenic <i>E. coli</i> (no. of isolates)			
	EAEC (13)	ETEC (5)	EPEC (6)	EIEC (4)
12:0, ALDE	2.38	2.85	1.92	2.44
12:0	3.79	3.28	3.60	3.15
14:0	7.43	7.13	8.71	5.82
15:0	1.84	4.65	3.76	8.51
3-OH -14:0	6.03	5.83	6.26	5.73
16:1, ω 7c	4.86	13.48	5.99	11.75
16:0	34.35	25.32	33.04	27.91
17:0, cyclo	20.45	11.33	20.31	12.25
17:0	1.51	3.06	1.34	4.45
18:1	5.88	11.95	6.79	12.84
18:0	1.45	1.33	1.16	1.45
19:0, cyclo ω 8c	10.15	9.08	6.17	2.36

Abbreviations: EAEC = enteroaggregative *E. coli*; ETEC = enterotoxigenic *E. coli*; EPEC = enteropathogenic *E. coli*; EIEC = enteroinvasive *E. coli*; ALDE = aldehydes

O111, and O119 are now rare, while enteroadherent strains other than EPEC, belonging to serogroups such as O125, were prevalent among the 126 diarrheic infants less than 1 year old who were surveyed [21].

Since we did not perform functional assay of the isolates, it is possible that strains which did not express the virulence genes or exhibit sequence variation in the primer binding sites were not detected. It has also been reported that about 10% of strains with an aggregative pattern in the HEP-2 cell assays do not harbor the *aggR* gene [18]. Molecular studies are needed to identify sequences specific for these strains in order to fill this information gap.

CFA profiles of bacteria for differentiation between toxigenic and non-toxigenic strains of *Clostridium difficile* have been reported [22]. CFA profiling has also been used for evaluating outbreaks caused by various bacteria, including group A *Streptococcus* [23]. This study showed that CFA data could be used to reliably identify *E. coli* isolates to the species level using MIDI Microbial Identification System software. Further comparison of CFA composition revealed minor variation in the percentage of each fatty acid among DEC isolates of ETEC, EPEC, EIEC and EAEC, which was not sufficient for differentiating between categories of DEC.

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