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Detection and Characterization of Diarrheagenic *Escherichia coli* from Young Children in Hanoi, Vietnam

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Diarrhea continues to be one of the most common causes of morbidity and mortality among infants and children in developing countries. *Escherichia coli* is an emerging agent among pathogens that cause diarrhea. The development of a highly applicable technique for the detection of different categories of diarrheagenic *E. coli* is important. We have used multiplex PCR by combining eight primer pairs specific for enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli*, enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC). This facilitates the identification of five different categories of diarrheagenic *E. coli* from stool samples in a single reaction simultaneously. The prevalences of diarrheagenic *E. coli* were 22.5 and 12% in the diarrhea group and the control group, respectively. Among 587 fecal samples from Vietnamese children under 5 years of age with diarrhea, this technique identified 132 diarrheagenic *E. coli* strains. This included 68 samples (11.6%) with EAEC, 12 samples (2.0%) with EIEC, 39 samples (6.6%) with EPEC, and 13 samples (2.2%) with ETEC. Among the 249 age-matched controls, 30 samples were positive for diarrheagenic *E. coli*. The distribution was 18 samples (7.2%) with EAEC, 11 samples (4.4%) with EPEC, and 1 sample (0.4%) with ETEC.

Diarrheal diseases are major causes of morbidity, with attack rates ranging from 2 to 12 or more episodes per person per year, especially in developing countries. In addition, diarrheal illnesses account for an estimated 12,600 deaths each day in children under 5 years of age in Asia, Africa, and Latin America. The causes of diarrhea include a wide range of viruses, bacteria, and parasites (14). Among the bacterial pathogens, *Escherichia coli* plays an important role. *E. coli* is the predominant nonpathogenic facultatively anaerobic member of the human intestinal microflora. Some *E. coli* strains, however, have developed the ability to cause diseases of the gastrointestinal, urinary, and central nervous systems in the human host. *E. coli* has been implicated as an agent of diarrheal disease since the 1920s (26). Diarrheagenic strains of *E. coli* can be divided into five main categories on the basis of distinct epidemiological and clinical features, specific virulence determinants, and association with certain serotypes (20): enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC). Some assays for the detection of diarrheagenic *E. coli* are available, such as biochemical reactions, serotyping, phenotypic assays based on virulence characteristics, and molecular detection methods (25). Among these, PCR, one of the molecular biology-based detection methods, is a commonly used method that gives rapid, reliable results and that also has a high sensitivity and a high specificity (5, 38). In order to detect five different categories of diarrheagenic *E. coli* from stool samples, it is necessary to run several individual PCRs with different primer pairs. In order to simplify diagnosis, we set up a multiplex PCR by combining eight primer pairs specific for EAEC, EIEC, EHEC, EPEC, and ETEC to detect these types of *E. coli* strains simultaneously in a single reaction.

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MATERIALS AND METHODS

Clinical specimens. During the period from March 2001 to April 2002, a total of 836 children from 0 to 60 months of age were studied. This included 587 children with diarrhea attending three different hospitals and 249 age-matched controls attending one day care center and one health care center in Hanoi, Vietnam. Information about some epidemiological factors was obtained through questionnaires. Patients were enrolled in the study if they had diarrhea, characterized by the occurrence of three or more, loose, liquid, or watery stools or at least one bloody loose stool in a 24-h period. An episode was considered resolved on the last day of diarrhea, followed by at least 3 diarrhea-free days. An episode was considered persistent if it continued for ≥14 days (4). Control subjects were healthy children (with no history of diarrhea for at least 1 month). Stool samples collected in Cary-Blair transport medium were cultured on the surface of sorbitol MacConkey agar (SMAC; Labora, Stockholm, Sweden) for the selection of *E. coli* isolates and on other media, such as TCBS Cholera Medium (Labora) for the selection of *Vibrio* and deoxycholate citrate agar (Sigma-Aldrich, Stockholm, Sweden) for the selection of *Shigella* and *Salmonella*, followed by overnight incubation at 37°C. All the samples were tested for *Vibrio*, *Shigella*, and *Salmonella* by using colony morphology, biochemical properties, and agglutination with specific sera. The multiplex PCR used for identification of diarrheagenic *E. coli* is described below.

Bacterial strains. The reference strains used for PCR are listed in Table 1. We used 238 verified strains of different *E. coli* categories from the Swedish Center for Control of Infectious Diseases, Culture Collection of University of Gothenburg, and the strain collection at the Division of Clinical Bacteriology (Karolinska University Hospital, Karolinska Institutet) to evaluate the multiplex PCR assay. These included 139 nondiarrheagenic *E. coli* isolates and 99 diarrheagenic *E. coli* isolates, divided into 18 strains of EAEC, 17 strains of EIEC, 15 strains of EHEC, 17 strains of EPEC, and 32 strains of ETEC.

Development of multiplex PCR for diarrheagenic *E. coli*. (i) Preparation of

TABLE 1. Reference *E. coli* strains for PCR

Category	Reference strain	Target gene(s)
ETEC	ATCC 35401	*eltB*, *estA*
EHEC	ATCC 43890	*vt1*, *eaeA*
EHEC	ATCC 43889	*vt2*, *eaeA*
EPEC	ATCC 43887	*eaeA*, *bfpA*
EIEC	ATCC 43893	*ial*
EAEC	97R[a]	pCVD432-harboring strain
E. coli (negative control)	ATCC 11775	No virulence gene

[a] The identity of this strain was verified by other methods; the strain was kindly provided by the Collaboration Project between Sweden and Nicaragua on Diarrhea.

DNA templates for PCR. A smear of bacteria from the first area of a SMAC plate was inoculated into a tube with 5 ml of phosphate-buffered saline to a density of a 4 McFarland standard (1×10^9 to 5×10^9 bacteria/ml). The 5-ml tube was boiled for 20 min, followed by centrifugation at 2,500 × *g* (Sorvall RT 6000 B refrigerated centrifuge; Dupont, Wilmington, Del.) for 10 min to pellet the cell debris. The supernatant was used for PCR.

(ii) Multiplex PCR assay. The DNA templates were subjected to multiplex PCR with specific primers (Table 2), as described previously (40), for the detection of the following virulence markers: *eaeA* (structural gene for intimin of EHEC and EPEC), *bfpA* (structural gene for the bundle-forming pilus of EPEC), *vt1* and/or *vt2* (Shiga toxins 1 and 2 of EHEC), *eltB* and/or *estA* (enterotoxins of ETEC), *ial* (invasion-associated locus of the invasion plasmid found in EIEC and *Shigella*), and pCVD (the nucleotide sequence of the EcoRI-PstI DNA fragment of pCVD432 of EAEC). The sequences of the primers selected for use in the amplification completely matched the sequences of the corresponding genes of EAEC, EHEC, EIEC, EPEC, and ETEC in the GenBank and EMBL database libraries.

The minimum criteria for determination of diarrheagenic *E. coli* were defined as follows: the presence of *eltB* and/or *estA* for ETEC, the presence of *vt1* and/or *vt2* for EHEC (the additional presence of *eaeA* confirms the detection of a typical EHEC isolate), the presence of *bfpA* and *eaeA* for typical EPEC (but the presence of only *eaeA* for atypical EPEC), the presence of *ial* for EIEC and *Shigella*, and the presence of pCVD for EAEC.

PCRs were performed with a 20-μl reaction mixture containing 2 μl of template DNA, 2 μl of 10× PCR buffer II, 1.6 μl of a 1.25 mM mixture of deoxynucleoside triphosphates, 1.6 μl of 25 mM $MgCl_2$, 0.1 μl of 5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer) per μl, and a 0.2 μM concentration of each primer except primer VT1 (all primers were from INTERACTIVA Biotechnologie GmbH, Ulm, Germany), which was used at a concentration of 0.4 μM. The thermocycling conditions with a Gene Amp PCR system 9700 (AB Applied Biosystem) were as follows: 96°C for 4 min, 94°C for 20 s, 55°C for 20 s, and 72°C for 10 s for 30 cycles, with a final 7-min extension at 72°C. PCR products (10 μl) were evaluated with a 1.5% (wt/vol) agarose gel (Gibco Life Technologies, Paisley, United Kingdom) at 120 mV for 30 min. A molecular marker (1-kb DNA ladder; Gibco/BRL) was run concurrently. The DNA bands were visualized and photographed under UV light after the gel was stained with ethidium bromide. First, multiplex PCR was performed with each reference strain of diarrheagenic *E. coli* from pure cultures (Fig. 1). Next, in order to

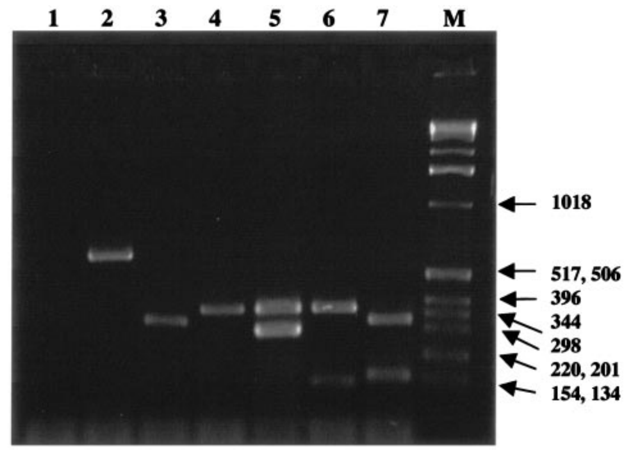

FIG. 1. Multiplex PCR amplification of reference strains of diarrheagenic *E. coli* from pure cultures. Lane 1, *E. coli* ATCC 11775; lane 2, EAEC 97R; lane 3, EIEC ATCC 43893; lane 4, EPEC ATCC 43887; lane 5, EHEC ATCC 43889; lane 6, EHEC ATCC 43890; lane 7, ETEC ATCC 35401; lane M, marker (1-kb DNA ladder; Gibco/BRL). Numbers on the right are in base pairs.

TABLE 2. Primers used in the multiplex PCR

Primer	Target gene	GenBank/EMBL accession no. or reference	Primer sequence	Amplimer size (bp)
LT	*eltB*	S60731	5′-TCTCTATGTGCATACGGAGC-3′ 5′-CCATACTGATTGCCGCAAT-3′	322
ST	*estA*	M34916	5′-GCTAAACCAGTA$^{G}{}_{A}$GGTCTTCAAAA-3′ 5′-CCCGGTACA$^{G}{}_{A}$GCAGGATTACAACA-3′	147
VT1	*vt1*	AF461172	5′-GAAGAGTCCGTGGGATTACG-3′ 5′-AGCGATGCAGCTATTAATAA-3′	130
VT2	*vt2*	AY143337	5′-ACCGTTTTTCAGATTTT$^{G}{}_{A}$CACATA-3′ 5′-TACACAGGAGCAGTTTCAGACAGT-3′	298
eae	*eaeA*	AE005595	5′-CACACGAATAAACTGACTAAAATG-3′ 5′-AAAAACGCTGACCCGCACCTAAAT-3′	376
SHIG	*ial*	12	5′-CTGGTAGGTATGGTGAGG-3′ 5′-CCAGGCCAACAATTATTTCC-3′	320
bfpA	*bfpA*	U27184	5′-TTCTTGGTGCTTGCGTGTCTTTT-3′ 5′-TTTTGTTTGTTGTATCTTTGTAA-3′	367
EA	pCVD	X81423	5′-CTGGCGAAAGACTGTATCAT-3′ 5′-CAATGTATAGAAATCCGCTGTT-3′	630

determine the detection limit of the multiplex PCR, stool samples negative for diarrheagenic *E. coli* were spiked with a phosphate-buffered saline suspension of reference diarrheagenic *E. coli* strains in serial 10-fold dilutions to give 10^0 to 10^8 CFU/ml. Each serial dilution of the spiked stool sample was spread onto an SMAC plate, and the plate was incubated at 37°C overnight. Multiplex PCR was performed with each dilution of spiked stool sample. The sensitivity of the assay was defined as the lowest concentration of diarrheagenic *E. coli* that yielded positive results for each dilution. There may be a possibility that two diarrheagenic *E. coli* strains are in the same sample. We therefore mixed each pair of two reference strains of diarrheagenic *E. coli* with nondiarrheagenic *E. coli* stool samples and carried out the multiplex PCR to detect these *E. coli* strains. Lastly, to confirm the detection of *E. coli* strains by the multiplex PCR and the specificity of the multiplex PCR, a collection of 238 strains verified to be of the different *E. coli* categories was tested. Standard precautions against carryover contamination were used (33).

Application of multiplex PCR for detection and identification of diarrheagenic *E. coli* strains from stool samples. The stool samples collected were inoculated onto the surfaces of SMAC plates. After incubation at 37°C overnight, a smear from the first area of a SMAC plate was taken for DNA extraction, as described above. The DNA template was subjected to the multiplex PCR. If the result was negative, the sample was considered negative for diarrheagenic *E. coli*. If the multiplex PCR was positive, the sizes of the bands on the gel were compared with those of the marker bands in order to identify certain kinds of diarrheagenic *E. coli* strains in the stool sample. Another smear of the same area was taken and cultured on a fresh SMAC plate to get separate colonies. After incubation at 37°C overnight, 5 to 10 colonies with typical *E. coli* morphologies were streaked onto fresh plates. Each colony was independently tested by PCR with a primer specific for a suspected diarrheagenic *E. coli* isolate from the multiplex PCR. Stocks of each isolate were kept at −70°C for further characterization.

Data analysis. The chi-square test was used to determine the statistical significance of the data. A *P* value of <0.05 was considered significant.

RESULTS

Multiplex PCR. (i) Multiplex PCR for reference strains. In this study, in order to detect five different categories of diarrheagenic *E. coli* simultaneously, a mixture of eight primer pairs specific for the target genes was used in a single PCR. Figure 1 (lanes 2 to 7) shows the PCR products derived from pure cultures of reference strains of EAEC, EIEC, EPEC, EHEC (*vt2*, *eae*), EHEC (*vt1*, *eae*), and ETEC, respectively. However, the band seen in lane 4 (EPEC) contains two bands, of 376 bp (*eae*) and 367 bp (*bfp*), so close together that it was impossible to separate them by agarose gel electrophoresis. Therefore, when the multiplex PCR was positive for EPEC from stool samples, it was necessary to run the specific PCRs separately with primers specific for *eae* and *bfp* to determine whether the strain was typical or atypical EPEC. The samples spiked with reference strains gave similar results.

(ii) Sensitivity of multiplex PCR. The sensitivity of the diagnostic multiplex PCR method was determined from the number of diarrheagenic *E. coli* cells (in CFU per milliliter) spiked into each milliliter of stool sample that could be detected by this method. Repeated experiments confirmed that the limit of detection of diarrheagenic *E. coli* was approximately 10^3 CFU/ml of stool suspension. Figure 2 shows the limit of detection of EHEC (*vt1* and *eaeA*) by multiplex PCR (the results for the other diarrheagenic *E. coli* strains are not shown).

(iii) Detection of two diarrheagenic *E. coli* strains in spiked stool samples by multiplex PCR. Stool samples spiked with each pair of the five different categories of diarrheagenic *E. coli* were tested by the multiplex PCR. The results in Fig. 3 show that the multiplex PCR could detect two diarrheagenic *E. coli* strains in a spiked stool sample. Due to the overlaps of some identical genes or close similarities in band sizes, it was difficult to distinguish individual bands in some cases. It was necessary to run the specific PCRs to verify the detection of these diarrheagenic *E. coli* strains. The nonspiked fecal sample was negative.

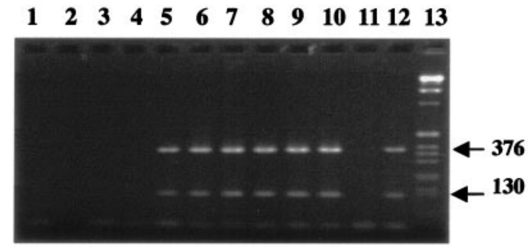

FIG. 2. Detection limit of EHEC (*vt1*, *eaeA*) from spiked stool sample. Lane 1, nonspiked stool sample; lanes 2 to 10, dilutions from 10^0 to 10^8 CFU/ml, respectively; lane 11, negative control; lane 12, positive control; lane M, marker (1-kb DNA ladder; Gibco/BRL). The 376- and 130-bp products (arrows on the right) correlate with the *eaeA* and *vt1* genes, respectively.

(iv) Multiplex PCR results for the 238 *E. coli* strains tested. Table 3 shows the results of the multiplex PCR with the 238 strains of various categories of *E. coli* tested. The multiplex PCR showed positive results for the diarrheagenic *E. coli* strains and negative results for the nondiarrheagenic strains.

Detection and characterization of diarrheagenic *E. coli* from clinical stool samples. A total of 162 diarrheagenic *E. coli* strains were isolated from 587 stool samples from children with diarrhea and from 249 samples from children in the healthy group. The prevalence of diarrheagenic *E. coli* in both groups was significantly different ($P < 0.001$). The PCR assays detected 68 (11.6%) EAEC isolates (pCVD PCR positive), 12 (2%) EIEC isolates (*ial* PCR positive), 39 (6.6%) EPEC isolates (*eae* PCR positive), and 13 (2.2%) ETEC isolates (*eltB* and/or *estA* PCR positive) from the group with diarrhea. The

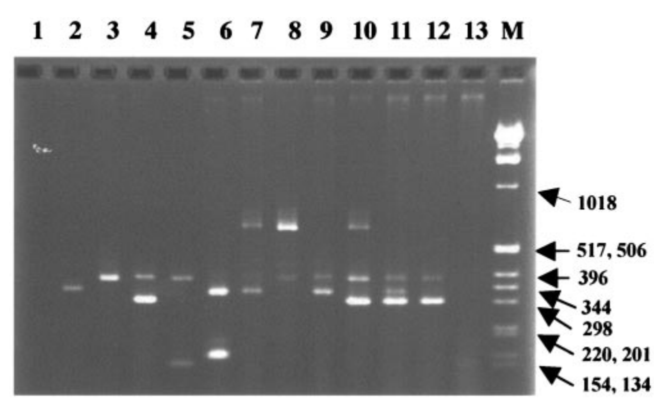

FIG. 3. Multiplex PCR amplification of each pair of two reference strains of diarrheagenic *E. coli* spiked with normal stool samples. Lane 1, negative control; lane 2, EIEC ATCC 43893; lane 3, EPEC ATCC 43887; lane 4, EHEC ATCC 43889; lane 5, EHEC ATCC 43890 and EPEC ATCC 43887; lane 6, ETEC ATCC 35401 and EIEC ATCC 43893; lane 7, EIEC ATCC 43893 and EAEC 97R; lane 8, EPEC ATCC 43887 and EAEC 97R; lane 9, EPEC ATCC 43887 and EIEC ATCC 43893; lane 10, EAEC 97R and EHEC ATCC 43889; lane 11, EHEC ATCC 43889 and EIEC ATCC 43893; lane 12, EHEC ATCC 43889 and EPEC ATCC 43887; lane 13, nonspiked stool sample; lane M, marker (1-kb DNA ladder; Gibco/BRL). Numbers on the right are in base pairs.

TABLE 3. Results of multiplex PCR with 238 verified *E. coli* strains

E. coli strain and gene	No. of strains		
	Total	Multiplex PCR	
		Positive	Negative
Diarrheagenic *E. coli* (n = 99)			
EAEC	18	18	0
EIEC	17	17	0
EHEC *vt1*	1	1	0
vt2	7	7	0
vt1 + *eae*	2	2	0
vt2 + *eae*	5	5	0
EPEC *eae*	4	4	0
eae + *bfp*	13	13	0
ETEC *elt*B	10	10	0
estA	14	14	0
eltB + *estA*	8	8	0
Nondiarrheagenic *E. coli* (n = 139)	139	0	139

prevalences of EAEC, EPEC, and ETEC in the healthy controls were 18 (7.2%), 11 (4.4%), and 1 (0.4%) isolates, respectively. No EHEC strains were isolated from any of the children tested (Table 4).

Of the ETEC strains isolated, 7 of 14 (50%) produced heat-labile toxin only, 4 of 14 (28.6%) produced heat-labile toxin and heat-stable toxin, and 3 of 14 (21.4%) produced heat-stable toxin only. All EPEC strains isolated were atypical. In the diarrhea group, EAEC and EPEC were more frequently isolated from children less than 2 years of age (14.1 and 7.9%, respectively), whereas EIEC and ETEC were less frequently found (1.9 and 1%, respectively) (Fig. 4). The prevalences of diarrheagenic *E. coli* in the healthy group were 5.9, 5, and 0.8% for EAEC, EPEC, and ETEC, respectively, in children from 0 to 24 months of age. Only EAEC and EPEC were isolated from the older children (8.5 and 3.8%, respectively). No significant differences in the prevalence of isolation of diarrheagenic *E. coli* by age were seen in the healthy group.

TABLE 4. Diarrheagenic *E. coli* strains isolated from stool samples

Diarrheagenic *E. coli*	PCR type	Total no. of samples (%) positive from children:		P
		With diarrhea (n = 587)	Without diarrhea (n = 249)	
EAEC	pCVD	68 (11.6)	18 (7.2)	0.057
EIEC	*ial*	12 (2.0)	0 (0)	<0.05
EPEC	*eae*	39 (6.6)	11 (4.4)	>0.05
ETEC	All	13 (2.2)	1 (0.4)	0.076
	eltB	7 (1.2)	0	0.11
	estA	4 (0.7)	0	0.32
	eltB-estA	3 (0.3)	1 (0.4)	1.00
Total		132 (22.5)	30 (12.0)	<0.001

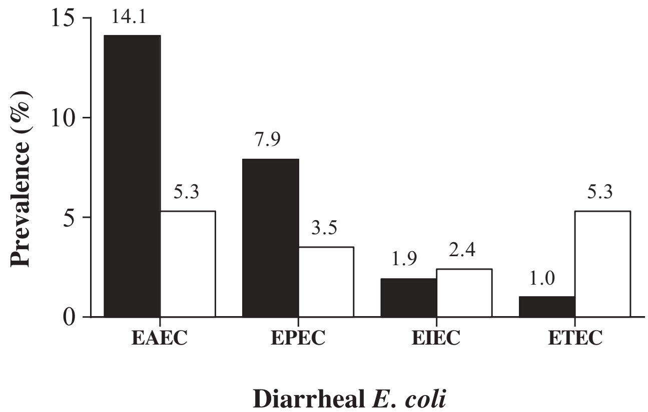

FIG. 4. Prevalence of diarrheagenic *E. coli* in the diarrhea group by age group. Significant differences in the prevalence of EAEC ($P < 0.005$) and ETEC ($P < 0.005$) were seen. Closed bars, children ages 0 to 24 months; open bars, children ages 25 to 60 months.

Twenty-eight *Shigella* strains, including 1 *Shigella boydii* strain, 7 *S. flexneri* strains, and 20 *S. sonnei* strains, were isolated from the diarrhea group. The prevalences of isolation of *S. sonnei* from children less than 2 years of age and the older children were 1.4 and 8.2%, respectively. The difference was statistically significant ($P < 0.0001$).

No *Vibrio cholerae* or *Salmonella* spp. were isolated from the diarrhea or the control group.

DISCUSSION

PCR is a powerful molecular biology technique for the detection of target DNA in various clinical specimens and for the detection of many kinds of pathogens. It is not only highly sensitive and specific, but it also provides rapid and reliable results. For stool samples, it can help to distinguish diarrheagenic *E. coli* isolates from those of the normal flora. Diarrheagenic *E. coli* strains are classified into five main categories according to the presence of different virulence genes. In order to detect these categories of *E. coli* strains, it is necessary to perform several PCRs with different primers specific for these genes. In this study, we have used a multiplex PCR by combining eight primer pairs specific for EAEC, EIEC, EHEC, EPEC, and ETEC in a single reaction. The sample DNA was prepared simply by boiling the bacteria in water for 20 min, followed by a short centrifugation. The DNA for PCR can be prepared from pure or mixed cultures. We were able to detect pathogenic *E. coli* strains from pure cultures and from spiked stool samples using reference strains by the multiplex PCR. In theory, PCR can detect a single copy of the target gene after 30 to 40 cycles of amplification. It was estimated that the limit of detection of diarrheagenic *E. coli* by the multiplex PCR was approximately 10^3 CFU/ml of stool sample. It was also shown that the presence of two types of diarrheagenic *E. coli* in a stool sample could be detected by multiplex PCR. Many previous studies have shown the existence of mixed infections in stool samples from children with diarrhea, including a bacterium and a bacterium, a bacterium and a virus, etc. (3, 4, 9, 11, 24).

Our results confirm the possibility that multiple types of diarrheagenic *E. coli* strains can be detected in a stool sample, as mentioned in other studies (2, 9, 32). This PCR was highly specific with the primers chosen for the detection of five categories of diarrheagenic *E. coli*. It showed positive results for the diarrheagenic *E. coli* strains tested and negative results for all nondiarrheagenic *E. coli* strains, indicating the high degree of specificity of the assay. In the case of *Shigella* spp., it is known that EIEC strains are closely related to *Shigella* in terms of their virulence and other phenotypic properties (19, 22). To date, PCR amplification assays based on *ipaH*, *ial*, and IS*630* have been used to detect *Shigella* and EIEC strains but cannot be used to differentiate between *Shigella* species and EIEC strains in human feces (12, 17, 36, 37). In this study, *ial*-specific PCR-positive strains were considered EIEC if they did not agglutinate with antisera specific to *Shigella* species. As a result of the similarity of the sizes of the DNA fragments amplified from *eaeA* and *bfpA* (376 and 367 bp, respectively) and from *eltB* and *ial* (322 and 320 bp, respectively) (Table 2), it was necessary to perform PCRs with specific primers after the multiplex PCR in order to verify the result. The samples could be processed within 1 day after collection and overnight growth, with a fraction of the labor and cost necessary for conventional assays.

In this study of children with diarrhea in Hanoi, Vietnam, diarrheagenic *E. coli* strains were recovered more often from children with diarrhea than from healthy controls ($P < 0.001$). The observed differences in the rates of recovery of the four categories of diarrheagenic *E. coli* (EHEC was not found in any of the samples) are shown in Table 4. The prevalence of ETEC (2.2% in the diarrhea group and 0.4% in the controls) was lower in our study than in some previous studies. According to Mayatepek et al. (23), the corresponding figures were 28 and 16%, respectively. The prevalence of ETEC was shown to be 20.7% in patients in other studies (23, 42, 43). However, other investigators have seen the same low prevalence of ETEC in children with diarrhea (27). Unlike the results of Albert et al. (2), the prevalence of ETEC in the diarrhea group in our study was significantly higher in children older than 2 years of age than in those younger than 2 years of age (Fig. 4) ($P < 0.005$). The prevalence of EIEC and *Shigella* has been reported to be different in different geographical areas (2, 4, 9, 29). The present study increased our understanding of the role of *Shigella* and EIEC in diarrhea in children. We isolated 28 *Shigella* spp. (4.7%) from the diarrhea group, with a significantly higher prevalence in children older than 2 years of age. The majority of these *Shigella* isolates were *S. sonnei*. In our diarrhea group, the prevalence of EIEC in children older than 2 years of age was not significantly different from that in children younger than 2 years of age (Fig. 4) ($P > 0.05$). Echeverria et al. (11) showed the opposite results. The prevalence of *Shigella* was even higher in children with dysentery (49% for *Shigella* and 6% for EIEC) in a study from Thailand conducted in 1989 and 1990 (10). In contrast to the relatively high prevalence of *Shigella* and EIEC in these studies, EIEC strains were not isolated and *Shigella* spp. were detected at a low prevalence in children under 5 years of age with diarrhea in studies in Bangladesh and Nigeria (2, 28). Besides ETEC and EIEC, EPEC is also a very important pathogen in children with diarrhea. The most notable feature of the epidemiology of the disease due to EPEC is the striking age distribution of the patients. EPEC infection is primarily a disease of infants younger than 2 years of age. As reviewed by Levine and Edelman (21), numerous case-control studies in many countries have found that EPEC is more frequently isolated from children with diarrhea than from healthy controls (25). In our study, the prevalence of isolation of EPEC strains from children with diarrhea was slightly higher than that from the controls (Table 4). Within the diarrhea group, the prevalences in children under 2 years of age and the older age group were 7.9 and 3.5%, respectively ($P = 0.053$). All the EPEC strains isolated were *eaeA* PCR positive but were negative for *bfpA*; i.e., they were all atypical EPEC isolates. *BfpA* is the structural gene encoding BFP (the bundle-forming pilus). These fimbriae are produced only under certain culture conditions (25). This could be the reason for the failure to identify them in our study, as shown by others (35). EPEC and EHEC share *eaeA* (the intimin structural gene), but the major virulence factor defining the characteristics of EHEC is Stx. In the present study, we did not isolate any EHEC strains from any of the groups of children. Similarly, no child with diarrhea was infected with EHEC in other studies (2, 30, 41), which concurs with the low prevalence of EHEC infection in the countries of the region evaluated in the present study (7, 39). Some studies have suggested that there is an interesting phenomenon in developing countries, in which EHEC is much less frequently isolated than other diarrheagenic *E. coli* strains, such as ETEC or EPEC strains (25). EAEC was the most commonly isolated category of diarrheagenic *E. coli* in both children with diarrhea and children without diarrhea. The prevalences were 11.6 and 7.2%, respectively (Table 4). A significantly higher prevalence of EAEC in the diarrhea group in children younger than 2 years of age was seen (Fig. 4) ($P < 0.005$). EAEC is an increasingly recognized cause of diarrhea worldwide, especially in developing countries (25, 26). An increasing number of studies support the association of EAEC with diarrhea in populations in developing countries, most prominently in association with persistent diarrhea (≥14 days). In several studies, culture of EAEC from the stool during the first few days of diarrhea is predictive of a longer duration of illness (8, 16). The association of EAEC with diarrhea appears to vary geographically, and many studies have demonstrated the importance of EAEC in pediatric diarrhea (6, 13, 18, 31). Our findings, however, were in contrast to the results of other investigators, who have not found EAEC to be associated with diarrhea (15, 34). One reason could be that the epidemiological characteristics of EAEC (e.g., likely sources, reservoirs of infection, routes of transmission, and seasonality) are largely unknown. In this study, EAEC was the most commonly isolated category of diarrheagenic *E. coli* from both the diarrhea and the control groups. EAEC has been known to cause persistent diarrhea in children in developing countries. It has also been recently reported to be a major etiologic agent in traveler's diarrhea in three regions of the world and an agent of diarrhea in immunocompromised patients (1, 25, 26). More studies are necessary to evaluate the contribution of EAEC to the human disease burden.

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