Diarrheagenic *Escherichia coli* and *Shigella* Strains Isolated from Children in a Hospital Case-Control Study in Hanoi, Vietnam[▽]

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Received 18 June 2007/Returned for modification 17 September 2007/Accepted 13 December 2007

This case-control study detected and characterized *Shigella* and diarrheagenic *Escherichia coli* (DEC) types among Vietnamese children less than 5 years old. In 249 children with diarrhea and 124 controls, *Shigella* spp. was an important cause of diarrhea ($P < 0.05$). We used multiplex PCR and DNA probes to detect enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC), enteropathogenic *E. coli* (EPEC), attaching and effacing *E. coli* (A/EEC), verocytotoxin-producing *E. coli* (VTEC), and enterotoxigenic *E. coli* (ETEC). The prevalences of DEC in the diarrhea and control groups were 25.7 and 10.5%, respectively. In 62 children with diarrhea, 64 DEC strains included 22 EAggEC (8.8%), 2 EIEC (0.8%), 23 A/EEC (9.2%), 7 EPEC (2.8%), and 10 ETEC strains (4.0%). Among controls, 13 DEC strains included 5 EAggEC strains (4.0%), 7 A/EEC strains (5.6%), and 1 EPEC strain. The characterization of DEC by serotypes, antimicrobial susceptibility patterns, virulence genes, and pulsed-field gel electrophoresis showed the occurrence of many different and highly heterogenic DEC subtypes, but common serotypes were found among ETEC, EIEC and EPEC, respectively. Serotyping was used to distinguish between A/EEC and EPEC. However, A/EEC, EPEC, and EAggEC were isolated at high frequency from both cases and controls. Further in-depth studies are needed to better understand important virulence factors of DEC, especially A/EEC, EPEC, and EAggEC.

Diarrheal disease is a major public health problem throughout the world, with over two million deaths occurring each year, mostly children under 5 years of age in developing countries (49). There is a wide range of recognized enteric pathogens such as viruses, bacteria, and parasites that cause diarrhea. Among bacteria, *Shigella* spp. and diarrheagenic *Escherichia coli* (DEC) are the most common causes of diarrheal diseases in developing countries (11).

DEC has been divided into six groups: verocytotoxin-producing *E. coli* (VTEC), attaching and effacing *E. coli* (A/EEC) including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC), and diffusely adherent *E. coli* (36). These *Shigella* and DEC groups have a variable importance of causing diarrhea, depending on geographical location and immune status of the host. *Shigella* globally has the highest incidence in children less than 5 years old and is the cause of 10% of all diarrheal episodes in this age group (11).

ETEC is one of leading causes of dehydrating diarrhea among weaning infants in the developing world. These children had two to three episodes of ETEC each of the first 2 years of life, which represents greater than 25% of all diarrheal illness and results in an estimated 700,000 deaths each year.

ETEC is also the major cause of travelers' diarrhea (11). VTEC is now recognized as a global health problem since it causes outbreaks around the world. This has mainly been documented in developed countries, and its effect in developing countries is unknown (11, 36). EIEC may have high prevalence in remote areas or cause outbreaks (20, 65). EAggEC is an increasingly recognized enteric pathogen and is the cause of acute or persistent diarrhea in children and adults in both developed and developing countries (36). A growing number of reports have described EAggEC outbreaks (19, 69, 72), and many reports have implicated EAggEC as a cause of sporadic diarrhea (41, 46). EPEC is an important cause of childhood diarrhea in developing countries (30, 48). However, the role of A/EEC and nonclassical EPEC O:H serotypes in childhood diarrhea is still questioned. Some nonclassical EPEC *eae*-positive serotypes have been reported to be associated with diarrhea (7, 17, 21, 47, 58) and may be named as "new EPEC." The prevalence and significance of new EPEC and A/EEC in childhood diarrhea is not well understood and seems to differ between countries (36). Some case-control studies have not been able to demonstrate any significant association between A/EEC and diarrhea (14, 52), although an association between A/EEC and prolonged diarrhea was found (2).

Etiological studies of diarrheal diseases have been done in Vietnam (25, 39), which have not identified all six DEC groups. Another study found that ETEC and EPEC were major causes of acute diarrhea, accounting for 25 and 45% of acute diarrhea pathogens in Tu Liem district and in a pediatric hospital, respectively (23). Nguyen et al. have identified all DEC groups

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[▽] Published ahead of print on 3 January 2008.

TABLE 1. Primers used for the detection of diarrheagenic *E. coli*

DEC type(s) [†]	Target gene	Primer	Sequence (5'-3')	Amplicon size in bp (reference)
ETEC	<i>eltB</i>	LT1	TCT CTA TGT GCA TAC GGA GC	322 (61)
		LTr	CCA TAC TGA TTG CCG CAA T	
VTEC	<i>estA</i>	ST12 l	GCT AAA CCA GTA G _A GG TCT TCA AAA	147 (61)
		ST12 r	CCC GGT ACA G _A GC AGG ATT ACA ACA	
	<i>vtx1</i>	VT1 l	GAA GAG TCC GTG GGA TTA CG	130 (61)
		VT1 r	AGC GAT GCA GCT ATT AAT AA	
EPEC	<i>vtx2</i>	VT2 l	ACC GTT TTT CAG ATT TTG _A CAC ATA	298 (61)
		VT2 r	TAC ACA GGA GCA GTT TCA GAC AGT	
	<i>eae</i>	eae u	CAC ACG AAT AAA CTG ACT AAA ATG	376 (61)
		eae l	AAA AAC GCT GAC CCG CAC CTA AAT	
EIEC and <i>Shigella</i> spp.	<i>bfpA</i>	bfp A2 u	TTC TTG GTG CTT GCG TGT CTT TT 3	367 (61)
		bfp A2 l	TTT TGT TTG TTG TAT CTT TGT AA	
	<i>ipaH</i>	IpaH III	GTT CCT TGA CCG CCT TTC CGA TAC CGT C	620 (57)
		IpaH IV	GCC GGT CAG CCA CCC TCT GAG AGT AC	
EAggEC	<i>aatA</i>	EA1	CTG GCG AAA GAC TGT ATC AT	630 (54)
		EA2	CAA TGT ATA GAA ATC CGC TGT T	

in one study in Hanoi (40). However, none of these studies included detailed characterization of DEC. In the present study, we sought to characterize DEC and *Shigella* species isolated from children in Hanoi, Vietnam, and to determine the role of DEC strains in diarrheal diseases.

MATERIALS AND METHODS

Specimen collection. During the period from May 2001 to May 2002, stools were collected from children aged 3 months to 5 years at the St. Paul Hospital in Hanoi. Written informed consent was obtained from the parents or guardian of each child. A total of 291 children with diarrhea and 291 age-matched controls were included in the original study (9). However, in our study only stool samples from 249 cases and 124 controls were analyzed for DEC and *Shigella* spp. due to limited resources available in the laboratory. Diarrhea was defined as (i) at least three loose (or watery) stools within 24 h, regardless of other gastrointestinal symptoms; (ii) two or more loose stools associated with at least one other symptom of gastrointestinal infection (abdominal pain, cramping, nausea, vomiting, and fever); or (iii) passage of a single loose stool with grossly evident blood and/or mucous (25). Two independent episodes were separated by at least 3 days of being diarrhea-free. An episode was considered persistent if it lasted for more than 14 days. Clinical symptoms such as nausea, vomiting, fever, and dehydration, as well as the medical history and previous treatment, were recorded on study forms. Children within the same age group who were seen at the same hospital for noninfectious causes and had no history of diarrhea in the preceding 2 weeks served as controls.

Microbiological analysis. Within the day a case or control was ascertained, stool samples were collected in plastic containers and Cary-Blair transport medium (Difco Laboratories, Detroit, MI). In the microbiological laboratory at St. Paul Hospital, stool samples were spread on MacConkey and Hektoen enteric agar plates (Difco Laboratories) and incubated at 37°C until transportation to the laboratory of the National Institute of Hygiene and Epidemiology (NIHE), in Hanoi, Vietnam, within the day of collection. Stools in Cary-Blair transport medium were stored at 4°C until being shipped at cold conditions to the Armed

Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand at the end of each week.

Shigella, *Salmonella*, *Vibrio*, and *Campylobacter* spp. were identified by standard microbiological methods at the laboratory of the NIHE during the day after sample collection. All enteric pathogens isolated by the NIHE were stored and subsequently confirmed by similar characterizations at the AFRIMS in Bangkok, Thailand (9, 68).

Characterization of diarrheagenic *E. coli* by multiplex PCR and dot blot hybridization. Up to five colonies from each sample were pooled in a multiplex PCR with eight different primers (Table 1) at the NIHE to identify the DEC type as previously described (22). The criteria for determining the different DEC types by PCR were as follows: the presence of *eltB* and/or *estA* genes for ETEC, the presence of *vtx1* and/or *vtx2* for VTEC, the presence of *eae* for A/EEC and EPEC, the presence of *bfpA* for typical EPEC plasmids, the presence of *ipaH* for EIEC and *Shigella*, and the presence of *aatA* (formerly CVD432) for EAggEC. If the pooled DNA template result was negative after gel electrophoresis, the sample was considered negative for DEC. If bands were seen after gel electrophoresis, the band sizes were compared to the sizes of marker bands to identify the DEC type. If a mixed bacterial culture was PCR positive then the DEC type was determined for individual *E. coli* isolates collected from the slant agars and subcultured onto MacConkey agar before PCR with single primer sets.

In parallel at the AFRIMS in Bangkok, stools in Cary-Blair transport medium were cultured, and enteric pathogens were identified by standard methods (9). In addition, up to five *E. coli* colonies per fecal sample were randomly picked and tested for DEC (ETEC, EIEC, A/EEC, EPEC, and VTEC) by the DNA hybridization technique (9) using specific digoxigenin-labeled DNA probes to detect *estA*_h, *estA*_p, *eltB*, *ial*, *eae*, *bfpA*, *vtx1* (SLTI), *vtx2* (SLTII), and plasmid marker CVD432 for EAggEC (Table 2). Further characterization at the AFRIMS included EPEC adherence factor EAF (15).

All DEC-positive strains from the NIHE and the AFRIMS were stored in soft nutrient agar at room temperature (6) until transfer to the Statens Serum Institut (SSI), Copenhagen, Denmark, took place. Confirmation of the DEC types by dot blot hybridization (Table 2) was done as previously described (22), and further characterization was done with the virulence genes and plasmids *astA* (51), *ehxA*

TABLE 2. Target genes for PCR, and DNA probes at AFRIMS and SSI

DEC type(s)	Target gene (PCR)	DNA probes/plasmid cloned of virulence determinant (reference)	DNA probes at SSI/plasmid cloned of virulence determinant (reference)
ETEC	<i>eltB</i>	<i>eltB</i> /D2232 (9)	<i>eltB</i> /DAS101 (59, 60)
VTEC	<i>estA</i>	<i>estA</i> _p /D2250), <i>estA</i> _h /D2232 (9)	<i>estA</i> _p /G119, <i>estA</i> _h /DAS100 (59, 60)
	<i>vtx1</i>	Shiga-like toxin I/pJN 37-19 (15)	<i>vtx1</i> /NTP705 (62)
	<i>vtx2</i>	Shiga-like toxin II/pJN 110-18 (15)	<i>vtx2</i> /DEP28 (62)
EPEC	<i>eae</i>	<i>eae</i> /pCVD 434 (28)	<i>eae</i> /pCVD 434 (28)
	<i>bfpA</i>	<i>bfpA</i> /pMSD 207 (16)	<i>bfpA</i> /pMSD 207 (16)
EIEC and <i>Shigella</i> spp.	<i>ipaH</i>	<i>ial</i> /pJM 17 (9)	<i>ipaH</i> /WR390 (63)
EAggEC	<i>aatA</i>	<i>aatA</i> /pCVD 432 (42)	<i>aatA</i> /pCVD 432 (42)

TABLE 3. Breakpoint values for MIC testing of different DEC types

Antimicrobial agent ^a	Breakpoint (μg/ml)	Test range (μg/ml)
AMP	32	1–32
AMC	32	2–32
APR	32	4–64
XNL	8	0.5–8
CEF	32	2–64
CHL	32	2–64
CIP	0.125/4	0.03–4
COL	16	4–64
FFN	32	2–64
GEN	8	1–32
NAL	32	8–128
SPT	128	4–128
NEO	16	2–32
STR	32	4–64
SMX	512	64–1024
TET	16	2–32
TMP	16	4–32
CPD	4	0.125–4

^a Abbreviations: APR, apramycin; CEF, cephalothin; COL, colistin; FFN, florfenicol; GEN, gentamicin.

(32), *daaC* (8), and EPEC adherence factor EAF (35). All confirmed dot blot-positive *E. coli* strains were further characterized as described below.

Serotyping. Identification of somatic (O) and flagellar (H) antigens was done by tube and microtiterplate agglutination with specific antisera O1–O181 supplemented with presumptive new O groups OX182–OX186 and H1–H56 as previously described (45). *E. coli* strains (*vtx* negative) reacting with the *eae* probe belonging to one of the classical EPEC serotypes (O26:NM, O26:H11, O26:H34, O55:NM, O55:H6, O55:H7, O86:NM, O86:H34, O111:NM, O111:H2, O111:H25, O114:NM, O114:H2, O119:NM, O119:H2, O119:H6, O125:H-, O125:H6, O125:H21, O126:NM, O126:H2, O126:H21, O126:H27, O127:NM, O127:H6, O126:H21, O128:NM, O128:H2, O128:H7, O128:H12, O142:NM, O142:H6, O158:NM, and O158:H23) were classified as EPEC (27). Serotypes O39:NM, O88:H25, O111:H8, O111:H9, O126:H12, O127:H4, O145:H45, O157:H8, and O157:H45 were considered new EPEC strains (27). Strains not belonging to these serotypes were classified as A/EEC. Strains reacting with EAF and/or *bfpA* were considered typical and, if they did not react with either of these two DNA probes, they were considered atypical.

Antimicrobial susceptibility testing by MIC. Antimicrobial susceptibility testing was done for 77 DEC and 22 *Shigella* spp. with Sensititre (Trek Diagnostic System, Ltd., East Grinstead, West Sussex, England), a commercially available MIC technique using dehydrated antimicrobials in microtiter wells. The wells were inoculated and incubated according to the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) (38). The MIC was defined as the lowest concentration of antimicrobial with no visible bacterial growth and the breakpoints used are shown in Table 3. The *E. coli* ATCC 25922 was used for quality control, and the MICs for the strains were evaluated in accordance to Clinical and Laboratory Standards Institute guidelines.

PFGE. The PulseNet method of the Centers for Disease Control and Prevention (49) was used for pulsed-field gel electrophoresis (PFGE) typing of the *E. coli* isolates. The XbaI restriction enzyme was used for genomic DNA digestion, and fragments obtained were resolved by using a contour-clamped homogeneous electric field CHEF-II Mapper (Bio-Rad, Hillerød, Denmark). DNA band patterns were visualized by UV illumination, photographed, analyzed, and compared using Gel Compar II software (Applied Maths, Kortrijk, Belgium). We used the band-based Dice similarity coefficient and a UPGMA (unweighted pair-group method with arithmetic averages) geometric matched analysis dendrogram type with a position tolerance setting of 1.5% for optimization and position tolerance of 1.5% for band comparison.

Data analysis. A total of 373 stool specimens (249 cases and 124 controls) out of 291 cases and 291 controls were analyzed for *Shigella* and DEC in the present study due to logistic matter. The chi-square test with Yates' correction or the Fisher exact test if any expected value were <5 was used to determine the statistical significance between case and control groups (EPI INFO version 6.04). A *P* value of <0.05 was considered significant.

TABLE 4. Isolation of bacterial pathogens from children with and without diarrhea

Pathogen	No. of positive (no. at NIHE):		<i>P</i>
	Cases (<i>n</i> = 249)	Controls (<i>n</i> = 124)	
DEC	64 (39)	13 (6)	0.001
EAggEC	22 (22)	5 (5)	0.10
EIEC	2 (2)	0 (0)	1
A/EEC	23 (0)	7 (0)	0.30
EPEC	7 (7)	1 (1)	0.28
ETEC	10 (6)	0	0.03
<i>Shigella</i> spp.	21 (22)	1 (1)	0.006

RESULTS AND DISCUSSION

Campylobacter and *Salmonella* spp. were found among 4 and 7% of the cases, respectively, and have been reported elsewhere (9).

***Shigella*.** Twenty-one *Shigella* strains were isolated, including *Shigella flexneri*, *S. dysenteriae*, and *S. sonnei* from the diarrhea group and one *S. flexneri* 2a from the control group. *Shigella* was the most important bacterial pathogen associated with diarrhea (Table 4; *P* < 0.05), accounting for 8.5% of the cases with diarrhea. This is similar to other findings (3, 44, 66, 70). Clinically, the infection was characterized by fever (62%), 48% had watery diarrhea and vomiting (see Table 6). The prevalence of *S. sonnei* in children less than two years of age was 4.2% compared to 21% in the older children (*P* < 0.05).

Genotypes of DEC. *E. coli* colonies from a total of 373 stool specimens (249 cases and 124 controls) were available to identify DEC by multiplex PCR at the NIHE in Hanoi. Multiplex PCR yielded 45 positive specimens. Dot blot hybridization at the AFRIMS yielded additional 32 positive samples (28 *eae* and 4 ETEC), giving a total of 77 samples with DEC. Dot blot hybridization performed at the SSI of these 77 positive samples confirmed the combined characterizations done at NIHE and AFRIMS (Table 5). All statistical analyses for DEC were based on these results.

The use of CVD434 for the *E. coli* attaching-and-effacing gene *eae* hybridization with 1-kb product (28) could be the reason for the higher number of *eae*-positive samples in dot blot hybridization at AFRIMS compared to PCR at the NIHE. A search of GenBank indicated that variants of intimin *eae* genes: β1, γ1, ε1, μR/ι2, o, νB, and ξB shared sequence homology of only 60 to 90% with the primer pairs used in the NIHE multiplex PCR (alignment applied by www.ebi.ac.uk/emboss/align/index.html). Further analysis of the strains that were positive by dot blot hybridization and negative by PCR would require sequencing or further subtyping of the *eae* genes negative by PCR and is beyond the scope of the present study. However, the observed discrepancy illustrates the difficulties associated with the appropriate choice of PCR primers for genes where polymorphism is known to occur. The enterotoxins LT and ST of ETEC are encoded by genes located on plasmids (36). The reason for the discrepant results for ETEC could be loss of the virulence plasmids genes. This kind of loss is not a rare observation either during in vitro storage or even during in vivo experiments (10, 33, 34). Furthermore, the relatively comparison between multiplex PCR and dot blot hy-

TABLE 5. Pathogenic group, virulence genes, serotypes, and PFGE types of 77 *E. coli* strains isolated from diarrhea cases and controls

Case or control no.	Pathogenic group ^a	Virulence genes	Serotype ^b	PFGE pattern	Status
D2785	EAggEC	<i>aatA</i>	O59:H10	6	Diarrhea
D2786	EAggEC	<i>aatA</i>	O14:H30	NT ^c	Diarrhea
D2787	EAggEC	<i>aatA</i>	O12:H4	10	Diarrhea
D2788	EAggEC	<i>aatA</i>	O86:H47	11	Diarrhea
D2789	EAggEC	<i>aatA</i>	O176:H34	7	Diarrhea
D2790	EAggEC	<i>aatA</i>	O153:H4	4	Diarrhea
D2791	EAggEC	<i>aatA</i>	O176:H34	7	Diarrhea
D2793	EAggEC	<i>aatA</i>	O rough:H10	6	Diarrhea
D2795	EAggEC	<i>aatA</i>	O78:H2	12	Diarrhea
D2797	EAggEC	<i>aatA</i>	O rough:NM	13	Diarrhea
D2798	EAggEC	<i>aatA</i>	O rough:H10	5	Diarrhea
D2799	EAggEC	<i>aatA</i>	O109:H23	14	Diarrhea
D2800	EAggEC	<i>aatA</i>	O86:NM	15	Diarrhea
D2802	EAggEC	<i>aatA</i>	O rough:H31	16	Diarrhea
D2805	EAggEC	<i>aatA</i>	O25:H4	17	Diarrhea
D2806	EAggEC	<i>aatA</i>	O126:H51	18	Diarrhea
D2807	EAggEC	<i>aatA</i>	O127:NM	19	Diarrhea
D2814	EAggEC	<i>aatA</i>	O104:H4	20	Control
D2794	EAggEC	<i>aatA</i> , <i>astA</i>	O39:H49	21	Diarrhea
D2804	EAggEC	<i>aatA</i> , <i>astA</i>	O15:NM	5	Diarrhea
D2808	EAggEC	<i>aatA</i> , <i>astA</i>	O153:H4	4	Control
D2809	EAggEC	<i>aatA</i> , <i>astA</i>	O153:H4	4	Control
D2803	EAggEC	<i>aatA</i> , <i>astA</i> , DA	IF1:H1	22	Diarrhea
D2810	EAggEC	<i>aatA</i> , <i>astA</i> , DA	O25:H4	23	Control
D2811	EAggEC	<i>aatA</i> , <i>astA</i> , DA	O25:H4	24	Control
D2796	EAggEC	<i>aatA</i> , DA	O25:H4	25	Diarrhea
D2792	EAggEC	<i>aatA</i> , EAF	O181:NM	26	Diarrhea
D2747B	A/EEC	<i>bfpA</i>	O21:H10	27	Diarrhea
D2750B	A/EEC	<i>bfpA</i>	O153:H42	28	Diarrhea
D2744	A/EEC	<i>eae</i>	O45:H15	29	Diarrhea
D2747A	A/EEC	<i>eae</i>	O34:H31	30	Diarrhea
D2748	A/EEC	<i>eae</i>	O118:H5	9	Diarrhea
D2750A	A/EEC	<i>eae</i>	O21:H19	31	Diarrhea
D2751	A/EEC	<i>eae</i>	O rough:H7	32	Diarrhea
D2754	A/EEC	<i>eae</i>	O rough:H7	33	Control
D2833	A/EEC	<i>eae</i>	O34:H31	34	Diarrhea
D3044	A/EEC	<i>eae</i>	O88:H5	35	Control
D3048	A/EEC	<i>eae</i>	O51:H7	10	Diarrhea
D3051	A/EEC	<i>eae</i>	O3:H51	9	Diarrhea
D3052	A/EEC	<i>eae</i>	O rough:H16	37	Control
D3056	A/EEC	<i>eae</i>	O145:H34	38	Control
D3059	A/EEC	<i>eae</i>	O51:H7	10	Diarrhea
D3061	A/EEC	<i>eae</i>	O34:H31	39	Control
D3062	A/EEC	<i>eae</i>	O132:H34	40	Diarrhea
D3068	A/EEC	<i>eae</i>	O145:H34	8	Diarrhea
D3070	A/EEC	<i>eae</i>	O145:H34	8	Control
D3076	A/EEC	<i>eae</i>	O33,O146:NM	41	Diarrhea
D3077	A/EEC	<i>eae</i>	O14:NM	42	Diarrhea
D3081	A/EEC	<i>eae</i>	O14:NM	43	Diarrhea
D3087	A/EEC	<i>eae</i>	O71:H40	44	Diarrhea
D3097	A/EEC	<i>eae</i>	O14:NM	45	Control
D3099	A/EEC	<i>eae</i>	O76:H5	46	Diarrhea
D3100	A/EEC	<i>eae</i>	O34:H31	47	Diarrhea
D3102	A/EEC	<i>eae</i>	O51:H7	48	Diarrhea
D2752	EPEC*	<i>eae</i>	O128ab:NM	49	Diarrhea
D3057	EPEC†	<i>eae</i>	O145:NM	50	Diarrhea
D3064	EPEC*	<i>eae</i>	O55:H7	51	Control
D3071	EPEC†	<i>eae</i>	O145:NM	52	Diarrhea
D3091	EPEC*	<i>eae</i>	O128ac:NM	53	Diarrhea
D3095	EPEC*	<i>eae</i>	O26:NM	54	Diarrhea
D3050	A/EEC	<i>eae</i> , <i>astA</i>	O88:H5	36	Diarrhea
D3066	A/EEC	<i>eae</i> , <i>astA</i>	OX186:NM	55	Diarrhea
D3080	A/EEC	<i>eae</i> , <i>astA</i>	O113:H19	56	Diarrhea
D3073	Typical EPEC	<i>eae</i> , <i>bfpA</i>	O26:NM	57	Diarrhea
D3046	Typical EPEC†	<i>eae</i> , <i>bfpA</i> , <i>astA</i>	O114:H25	58	Diarrhea
D2718	ETEC	<i>eltB</i>	O64:NM	1	Diarrhea
D2719	ETEC	<i>eltB</i>	O141ac:H10	2	Diarrhea
D2720	ETEC	<i>eltB</i>	O141ac:H10	2	Diarrhea
D2721	ETEC	<i>eltB</i>	O64:NM	1	Diarrhea
D2723	ETEC	<i>eltB</i>	O159:H34	59	Diarrhea
D2722	ETEC	<i>eltB</i> , <i>estAh</i>	O17:H18	60	Diarrhea
D3063	ETEC	<i>eltB</i> , <i>estAh</i> , <i>astA</i>	O6:H16	3	Diarrhea
D3096	ETEC	<i>eltB</i> , <i>estAh</i> , <i>astA</i>	O6:H16	3	Diarrhea
D3055	ETEC	<i>estAh</i> , <i>astA</i>	O25:NM	61	Diarrhea
D3069	ETEC	<i>estAh</i> , <i>astA</i>	O169:H41	NT	Diarrhea
D3053	EIEC	<i>ipaH</i>	O28ac:NM	62	Diarrhea
D3045	EIEC	<i>ipaH</i>	O136:NM	63	Diarrhea

^a *, classical serotypes belonged to a typical EPEC; †, new EPEC serotypes.^b NM, nonmotile; O rough, autoagglutinable.^c NT, not typeable by PFGE (the gel was smeared).

TABLE 6. Clinical symptoms associated with diarrheagenic *E. coli* and *Shigella* spp. isolated from cases of diarrhea

Pathogen (<i>n</i>) ^a	Age in mo (range)	No. (%) of children with:				
		Watery diarrhea	Bloody diarrhea	Fever	Pain	Vomiting
A/EEC (21)	10 (6–50)	15 (71)	0	11 (52)	6 (28)	14 (67)
EPEC (7)	10 (4–31)	4 (57)	0	4 (57)	4 (57)	4 (57)
EAggEC (22)	10 (3–21)	18 (89)	1 (4.5)	8 (36)	3 (13)	15 (68)
ETEC (10)	26 (6–48)	6 (60)	1 (10)	6 (60)	2 (20)	6 (60)
EIEC (2)	29 (20–38)	2 (100)	0	2 (100)	1 (50)	2 (100)
<i>Shigella</i> spp. (21)	22 (4–60)	10 (48)	4 (19)	13 (62)	7 (33)	10 (48)

^a *n*, Number of isolates.

bridization in Thailand was done on two independent sets of colonies. All DEC strains from Vietnam were confirmed by dot blot hybridization in Denmark, indicating no loss of genes. Detection of virulence genes could also depend on unequal amounts of colony culture in the pool or simply missing the DEC colony on the plate for the PCR.

DEC was recovered more often from 249 children with diarrhea than from 124 healthy controls ($P < 0.05$, Table 4). Two weeks were rather short for including healthy controls because it is not known how long the pathogens reside in the colon after an infection, but only EAggEC and A/EEC were found in controls. This emphasizes the importance of further studies on the persistence and colonization of these two DEC groups.

Most of the cases had watery diarrhea (73%). Half of the cases had fever (Table 6). The mean age of DEC-infected patients was 1 year. The rate of different DEC categories is shown in Table 4. A/EEC was the most frequently identified DEC type and were relatively more common in cases (9.2%) than in controls (5.6%). However, the identification of A/EEC at the present level using only the *eae* gene is clearly insufficient, and a much more in-depth characterization is warranted, together with information on clinical manifestation. Our finding is in contrast to findings from Australia, Iran, and Brazil, where A/EEC was associated with diarrhea (4, 44, 50), but in accordance with other studies (1, 2, 31, 36, 55). The *astA* was detected only in cases with A/EEC. This virulence marker may be a useful tool for diagnosing A/EEC pathogenic strains (13, 71). Classical EPEC serotypes were found more often among the cases (2.8%) than controls (0.8%), but the association with diarrhea was not statistically significant (Table 4). This is in contrast to a number of case-control studies which found that EPEC is associated with diarrhea (4, 12, 30, 36, 44). Only two *eae*-positive strains were positive for *bfpA*, a finding similar to what has been shown by others (56). EPEC and VTEC share *eae* but the main virulence factor to define VTEC is *vtx*. In the present study, we did not find any VTEC strains, which is similar to the findings of other studies (3, 41, 67). Some studies suggest that in developing countries VTEC is much less frequently isolated than other DEC strains, such as ETEC or EPEC (36). In the present study, ETEC was associated with diarrhea ($P < 0.05$), although the prevalence of only 4% in cases in the present study was lower than the 16 to 18% demonstrated by others (3, 64). However, other investigators have shown a similar low prevalence of ETEC in children with diarrhea (41, 44, 50). In addition to ETEC, EAggEC is an increasingly recognized cause of diarrhea worldwide, especially in developing countries (36, 37). In the present study, EAggEC

was not associated with diarrhea ($P = 0.1$). Several studies support this association (3, 41, 50), whereas others did not find EAggEC to be associated with diarrhea (18, 44, 53). The *aatA* (CVD432) is the standard gene probe to diagnose EAggEC, but it should be noted that the sensitivity of CVD432 varies from 15 to 90% in different locations (43) and that other genes, such as the *aggR* regulator, should also be used in order to detect a wider range of EAggEC.

We found a low frequency of EIEC; thus, EIEC cannot be considered a main cause of childhood diarrhea in Hanoi (44).

Serotyping. A total of 51 serotypes were isolated from the 59 cases and 13 controls (Table 5). Forty-one and ten serotypes were distributed among the cases and controls, respectively. Flagellum type H4 predominated in the controls (5 of 13), whereas H10 was observed only in cases. In 21 nonmotile strains, only one strain was isolated from a control. Two EIEC strains were isolated from diarrhea cases and belonged to classical EIEC serotype: O28ac:NM and O136:NM. Ten ETEC strains belonged to six common serotypes of human ETEC strains. Classical serotypes of EPEC were found in five cases (two O26:NM cases, two O128:NM cases, and one O114:H25 case) and an O55:H7 control. Two new EPEC serotypes O145:NM and O114:H25 were found in three cases. In contrast to the classical EPEC strains, the distribution of serotypes among A/EEC strains showed a great variation. A new O antigen (OX186) currently under investigation was found in a case of A/EEC. A high degree of diversity of O:H serotypes was observed among EAggEC from both cases and controls.

FIG. 1. Antimicrobial susceptibilities of DEC strains as determined by MIC testing. CEF, cephalothin.

FIG. 2. Dendrogram of 75 *E. coli* strains established based on PFGE typing patterns produced using band-based Dice similarity coefficient and UPGMA. D, diarrhea; C, control.

Antimicrobial susceptibility testing. The overall results from the antimicrobial susceptibility testing for DEC are shown in Fig. 1. Resistance to antimicrobials commonly used to treat diarrhea such as ampicillin (AMP), chloramphenicol (CHL), streptomycin (STR), tetracycline (TET), trimethoprim (TMP), and sulfamethoxazole (SMX) was observed in all DEC types. In addition, EAggEC strains were also resistant to other antimicrobials; three strains were resistant to nalidixic acid (NAL), one strain was resistant to neomycin (NEO), six strains were resistant to cefpodoxime (CPD), and five strains were resistant to ceftiofur (XNL). Resistance to ciprofloxacin (CIP) and NAL in DEC was 5%, which was lower than that determined in a recent study by Nguyen et al. (40), who found that 18.5% of DEC strains isolated in Vietnam were resistant to quinolones.

Nineteen of twenty-two *Shigella* strains (86%) were multiresistant to SMX, spectinomycin (SPT), STR, TET, and TMP; nine of these strains showed an additional resistance to AMP and CHL. Our study is in accordance with several other studies that reported multiresistance of *Shigella* to commonly used therapeutic antimicrobials (5, 25, 26, 40). Except for amoxicillin-clavulanic acid (AMC) exhibiting moderate activity to *Shigella*, other antibiotics were still active against this pathogen. Three remaining strains were sensitive to all tested antimicrobials.

There were no significant differences in antimicrobial resistance in DEC and *Shigella* strains from children with diarrhea compared to strains from control children (data not shown). The susceptibility testing with commonly used antimicrobials for diarrhea treatment (AMP, SMX, TMP, TET, and STR) resulted in MICs for strains determined as resistant twice as high as the breakpoint values (>32, >1,024, >32, >32, and >64 µg/ml, respectively), which showed a low activity of these antimicrobials against DEC and *Shigella* strains (40). However, in contrast to other studies (24, 40), the quinolones (e.g., NAL and CIP) can still be used for treatment in case the enteropathogens are resistant to traditionally used antibiotics. An immediate concern is the need for an effective and inexpensive antimicrobial agent that can be used safely for the treatment of children with diarrhea, especially in developing countries such as Vietnam.

PFGE. All of the isolates were analyzed by PFGE to assess their clonal relatedness. Two isolates could not be typed by PFGE; the rest showed 65 different patterns that differed by more than three bands (Fig. 2 and Table 5). Not surprisingly, strains of the same DEC pathotype and serotype were closely related since they were isolated only from cases with diarrhea. In accordance with our previous study (22), ETEC and EAggEC strains of the same serotype, same genotype, and same group of case or control showed high similarity in their PFGE patterns. For example, the similarity in PFGE patterns for the two cases with O64:NM *eltB* was 92%. The two cases with O6:H16 *astA*, *eltB*, and *estA* showed 91% similarity, the two cases with the O141ac:H10 *eltB* had 98% similarity, and the two controls O153:H4 *aatA* and *astA* had 92% similarity in PFGE patterns. Low similarity was, however, observed among A/EEC and EPEC strains of the same serotype and virulence type that occurred in both cases and controls. There were three cases and one control of O34:H31 *eae* (70%), and two cases and one control of O14:NM *eae* (80%). EAggEC and EPEC

strains of the same serotype but different virulence types also showed low similarity by PFGE, such as two cases of O25:H4 *aatA* and O25:H4 *aatA* and DA (65%), and two cases of O26:NM *eae* and O26:NM *eae bfpA* (71%) (Fig. 2). However, two EPEC strains, as well as six EAggEC strains, of different serotypes were closely related by PFGE. Two cases of O rough: H10 *aatA* and O15:NM *aatA* and *astA*, O59:H10 *aatA* and O rough:H10 *aatA*, and O118:H5 *eae* and O3:H51 *eae* were 90 to 94% similar by PFGE. Such similarities in PFGE patterns has been observed previously among EPEC, ETEC, and EAggEC strains in India (29).

In conclusion, the characterization of DEC strains showed a high level of resistance to commonly used antimicrobials for treatment of diarrhea and a high diversity of serotypes. Classical serotypes were found in EIEC, ETEC, and EPEC. In consideration of the result of serotypes, virulence genes, antimicrobial susceptibility, and PFGE patterns, our study showed that *E. coli* strains of the same pathotype or the same serotype are not monophyletic and do not cluster according to any of the studied features. Characterization of A/EEC, the most frequently identified of all DEC, did not reveal marked differences in serotypes, the presence of virulence genes, or antimicrobial resistance between cases and controls, except for the presence of *astA* in cases. Specific virulence genes for EPEC were not identified either. Therefore, serotyping is still necessary in order to distinguish between classical EPEC, new EPEC, and A/EEC. To our knowledge, this is the first report regarding clonal analysis using a molecular approach for DEC strains isolated from a hospital in Vietnam. However, molecular epidemiological studies in several locations and detailed characterization of DEC are required before we can arrive at any conclusion on their relative roles as causes of diarrhea. These studies are ongoing.

ACKNOWLEDGMENTS

This study received financial support from the Danish International Development Agency through the research capacity building project Sanitary Aspects of Drinking Water and Wastewater Reuse in Vietnam (grant 104.Dan.8.L), as well as the U.S. Army Medical Research and Materiel Command and the Department of Defense, Global Emerging Infections Surveillance and Response System.

We thank the clinicians, technicians, and staff of St. Paul Hospital, NIHE, in Hanoi and of AFRIMS in Bangkok for their microbiology expertise and assistance with enrollment, specimen collection, and processing. We also thank Susanne Jespersen, who helped with serotyping and dot blot hybridizations in Copenhagen. We especially thank Carl Mason at AFRIMS for his kind support in carrying out this study.

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