Diarrhoeagenic *Escherichia coli* and other causes of childhood diarrhoea: a case–control study in children living in a wastewater-use area in Hanoi, Vietnam

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A case–control study was conducted to identify the aetiology of diarrhoeal diseases in pre-school children in a suburban area of Hanoi where the use of untreated wastewater in agriculture and aquaculture is a common practice. Stool specimens and clinical information were collected from 111 pairs of children with diarrhoea and healthy controls. A total of 73 cases (66 %) and 41 controls (36 %) had an enteric pathogen. The pathogens most often associated with diarrhoea were rotavirus (17 % of cases) and *Entamoeba histolytica* (15 %), followed by *Shigella* (5 %). Diarrhoeagenic *Escherichia coli* (DEC) was found in 23 % of both patients and controls. Characterization of DEC by serotyping, antimicrobial susceptibility test and PFGE showed that DEC represented by different pathotypes belonged to various serotypes. Except for three enterotoxigenic *E. coli* strains, typing by PFGE revealed no correlation between pathotype and serotype of DEC strains. This suggests a high prevalence of a variety of DEC subtypes in this area. For this particular region, vaccine development strategies targeting rotavirus and *Shigella* are likely to be of public health benefit, whereas the role of DEC and preventive measures need to be further elaborated.

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INTRODUCTION

Diarrhoeal disease is a major problem throughout the world, and is responsible for high morbidity and mortality among children, especially in developing countries. Some aetiological studies of diarrhoeal diseases have been carried out in Vietnam (Isenbarger *et al.*, 2001; Nguyen *et al.*, 2005a), but not in areas where untreated wastewater is used in agriculture and aquaculture. The association of wastewater use and risks to human health has been assessed in various countries such as Israel, Morocco, Mexico and Pakistan, where wastewater is also commonly used for irrigation (Shuval *et al.*, 1989; Feenstra *et al.*, 2000; Habbari *et al.*, 2000; Blumenthal *et al.*, 2001; WHO, 2006). Some

studies have highlighted a high risk of being infected with intestinal parasites and of getting diarrhoeal diseases, especially in small children who live in the wastewater-using areas (Cifuentes, 1998; Cifuentes *et al.*, 2000).

In a hospital study, the prevalences of diarrhoeagenic *Escherichia coli* (DEC) were 22.5 and 12 % in the diarrhoea and control groups, respectively, but mainly due to a high frequency of enteroaggregative *E. coli* (EAaggEC) (Nguyen *et al.*, 2005a). Using dot-blot hybridization in another hospital-based study, *eae*-positive *E. coli* were found at a significantly higher prevalence in children with diarrhoea than in asymptomatic controls (Bodhidatta *et al.*, 2007). In a study outside Hanoi, *Campylobacter* and *Shigella* were found to be associated with diarrhoea, and enterotoxigenic *E. coli* (ETEC) was the prevalent group of DEC (Isenbarger *et al.*, 2001). None of these studies included detailed characterization of DEC.

The aim of the present study was to determine the aetiology of diarrhoeal diseases in children from families engaged in

Abbreviations: A/EEC, attaching and effacing *Escherichia coli*; DEC, diarrhoeagenic *E. coli*; EAF, EPEC adherence factor; EAaggEC, enteroaggregative *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; NIHE, National Institute of Hygiene and Epidemiology; VTEC, Vero cytotoxin-producing *E. coli*.

wastewater-irrigated agriculture or aquaculture activities in a suburban area of Hanoi, Vietnam. In particular, we aimed to determine the role of DEC by carrying out a detailed characterization.

METHODS

Study area. The study was conducted in Yen So commune (population 10 500), a south-east suburban area of Hanoi city with a long tradition for using untreated wastewater for irrigation in agriculture and for fishpond culture. Most of the city wastewater, mainly household sewage and industrial effluent, is discharged into three main canals, which run through this low-lying area before the wastewater reaches the recipient rivers. Wastewater-irrigated fish and vegetables are produced at low cost, seem widely accepted by the consumers and make a significant contribution to household economies (Hoan, 1996; Thang, 1996).

Study design and data collection. The parents of children under 72 months of age living in 400 randomly selected households were invited to let their children participate. This cohort of children was monitored from the middle of November 2002 to the end of May 2004 by weekly recall interviews. Trained field workers collected data on episodes of diarrhoeal disease, including date of onset, length of the episode, symptoms and treatment. An episode of acute diarrhoea was defined as: (i) at least three or more loose (or watery) stools within a 24 h period, regardless of other gastrointestinal symptoms; or (ii) two or more loose stools associated with at least one other symptom of gastrointestinal infection (abdominal pain, cramping, nausea, vomiting or fever); or (iii) passage of a single loose stool with grossly evident blood/mucous (Isenbarger *et al.*, 2001). Two independent episodes were separated by at least 3 days that were diarrhoea-free. An episode of diarrhoea with a duration of 14 days or more was regarded as an episode of persistent diarrhoea (Mølbak *et al.*, 1997).

For every recruited case, a control was randomly selected among the children residing in the 400 houses. An eligible control was a member of the cohort, but not living in the same house with the case, and who had not had diarrhoea in the preceding 4 weeks. Similarly, a control could later become a case if he/she developed diarrhoea within the specified period.

Faecal specimen collection. On the day that a case or control was ascertained, a stool sample was collected from the child. Stools were collected in plastic containers for parasitological and viral analyses, and in Cary–Blair transport medium (Difco Laboratories) for bacteriological analyses. When a specimen was unavailable, we collected rectal swabs and transferred them to Cary–Blair transport medium. Samples were stored in a refrigerator at the communal health station until transportation in cold boxes to the laboratory of the National Institute of Hygiene and Epidemiology (NIHE), Hanoi, on the day of collection.

Microbiological analysis of stools. Stools were processed and analysed for enteric bacteria and protozoan parasites at the laboratory of the NIHE on the day of sample collection. Standard culture and identification methods were used to identify enteric pathogens (WHO, 1987). In brief, *Entamoeba histolytica*, *Giardia lamblia* and *Cyclospora* spp. were identified by direct microscopy of a wet mount. Samples suspected to be positive for *Cyclospora* were confirmed by fluorescent microscopy. Stool specimens were tested for rotavirus with the IDEIA ELISA kit (Dako) as described by the manufacturer. Stools from Cary–Blair transport medium were cultured on SSI enteric medium (Blom *et al.*, 1999) for the isolation of *E. coli*. Suspected *E.*

coli colonies from SSI medium were further identified by a few selected biochemical tests: use of Simmons citrate, gas and hydrogen sulfide production, and lactose and glucose fermentation. Colonies that grew in Simmons citrate and/or produced gas and hydrogen sulfide and/or did not ferment glucose were discarded. Hektoen enteric agar plates were used to isolate *Shigella* spp. and thiosulfate citrate bile sucrose agar plates for *Vibrio* spp. Faecal samples were also inoculated into selenite F and Doyle's enrichment broth prior to subculture on *Brucella* agar (5% sheep blood) and Hektoen agar plates for the selection of *Salmonella* and *Campylobacter*, respectively. All agar plates and enrichment broths were incubated at 37 °C for 18–24 h. The microbiological media used were either from Difco Laboratories or from Becton Dickinson, except for the SSI enteric medium (Statens Serum Institut).

Shigella spp., *Salmonella* spp. and *Vibrio* spp. were identified by biochemical tests and antiserum agglutination (all antisera were from S&A Reagents Lab). *Campylobacter* spp. were isolated by membrane filtration using cellulose triacetate membranes with a 0.45 µm pore size placed on *Brucella* agar (5% sheep blood; NIHE) plates, which were incubated at 42.0 ± 0.5 °C for 48 h under microaerophilic conditions (CampyGen CN25; Oxoid) (Steele & McDermott, 1984). The suspect colonies were examined by microscopy following Gram staining (Wang & Murdoch, 2004) and considered to be *Campylobacter* spp. when curve-shaped and motile bacteria were observed, and a positive catalase and oxidase reaction was found. *Campylobacter jejuni* and *Campylobacter coli* were differentiated based on the results of a hippurate hydrolysis test (Bolton *et al.*, 1992).

Characterization of DEC by multiplex PCR and dot-blot hybridization. Multiplex PCRs with eight different primers were carried out at the NIHE, Hanoi, to identify the type of DEC (and *Shigella*) (Table 1). The criteria for determining the different types of DEC by PCR were as follows: the presence of *eltB* and/or *estA* genes was used to detect ETEC, the presence of *vtx1* and/or *vtx2* to detect Vero cytotoxin-producing *E. coli* (VTEC), the presence of *eae* to detect attaching and effacing *E. coli* (A/EEC), the presence of *bfpA* to detect enteropathogenic *E. coli* (EPEC) plasmids, the presence of *ipaH* to detect enteroinvasive *E. coli* (EIEC) and *Shigella*, and the presence of *aatA* (formerly CVD432) to detect EAaggEC. Five to seven colonies selected from each primary plate were subcultured on nutrient slant agar (Difco) and incubated overnight at 37 °C. Bacterial cultures from the five to seven slant agars were suspended in PBS to 1 McFarland standard (10^8 bacteria ml⁻¹) and boiled for 10 min, followed by centrifugation at 13 000 g for 5 min. Two microlitres of the DNA template was amplified in a final volume of 20 µl containing 0.5 mM each dNTP, 2 µl PCR buffer [150 mM Tris/HCl (pH 8.0), 500 mM KCl], 1.2 µl 25 mM MgCl₂, 1.6 µl each 2.5 µmol primer mix and 0.5 U *Taq* Gold DNA polymerase. The PCR was carried out in a DNA thermal cycler 480 (Perkin Elmer) with 30 cycles of 94 °C for 40 s, 53 °C for 1 min and 72 °C for 1 min. PCR products (10 µl) were then electrophoresed on 1.5% (w/v) agarose gel (Gibco Life Technologies) at 120 mV for 30 min and visualized with a UV transilluminator after ethidium bromide staining. If the pooled DNA template result was negative following gel electrophoresis, the sample was considered negative for DEC. If bands were seen after gel electrophoresis, the band sizes were compared with 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 (Difco) before PCR with single primer sets.

All PCR-positive strains were transferred to the Statens Serum Institut to verify the DEC type. Strains were examined for the presence of virulence genes using DNA probes derived from: NTP705, Vero cytotoxin 1 (*vtx1*) (Willshaw *et al.*, 1985); DEP28, Vero cytotoxin 2 (*vtx2*) (Thomas *et al.*, 1991); pSS126, the enteroaggregative heat stable

Table 1. PCR primers used for the identification of different types of DEC

DEC type	Target gene	Primer	Primer sequence (5'→3')	Amplicon size (bp)	Reference
ETEC	<i>eltB</i>	LT1	TCTCTATGTGCATACGGAGC	322	Svenungsson <i>et al.</i> (2000)
		LTr	CCATACTGATTGCCGCAAT		
VTEC	<i>estB</i>	STI2 l	GCTAAACCAGTAG _A GGTCTTCAAAA	147	Svenungsson <i>et al.</i> (2000)
		STI2 r	CCCGGTACAG _A GCAGGATTACAACA		
	<i>vtx1</i>	VT1 l	GAAGAGTCCGTGGGATTACG	130	Svenungsson <i>et al.</i> (2000)
		VT1 r	AGCGATGCAGCTATTAATAA		
EPEC	<i>vtx2</i>	VT2 l	ACCGTTTTTCAGATTTTG _A CACATA	298	Svenungsson <i>et al.</i> (2000)
		VT2 r	TACACAGGAGCAGTTTCAGACAGT		
	<i>eae</i>	eae u	CACACGAATAAACTGACTAAAAATG	376	Svenungsson <i>et al.</i> (2000)
		eae l	AAAAACGCTGACCCGCACCTAAAT		
EIEC	<i>bfpA</i>	bfp A2 u	TTCTTGGTGCTTGCGTGCTTTTT	367	Svenungsson <i>et al.</i> (2000)
		bfp A2 l	TTTTGTTTGTGTATCTTTGTAA		
	<i>ipaH</i>	IpaH III	GTTCCTTGACCGCCTTTCCGATACCGTC	620	Sethabutr <i>et al.</i> (1993)
		IpaH IV	GCCGGTCAGCCACCCTCTGAGAGTAC		
EAggEC	<i>aataA</i>	EA1	CTGGCGAAAGACTGTATCAT	630	Schmidt <i>et al.</i> (1995)
		EA2	CAATGTATAGAAATCCGCTGTT		

toxin (*astA*) (Savarino *et al.*, 1996); CVD419, the plasmid-encoded enterohaemolysin (*ehxA*) (Levine *et al.*, 1987); PS2.5, the invasive plasmid in EIEC (Small & Falkow, 1986); WR390, the invasion plasmid antigen gene *ipaH* found in EIEC and *Shigella* (Venkatesan *et al.*, 1989); CVD432, the plasmid marker *aataA* encoding a dispersin translocator (Nishi *et al.*, 2003) for EAggEC (Baudry *et al.*, 1990); SLM862, detecting the *daaC* gene from the *daa* locus encoding the afimbrial adhesin F1845, mediating diffuse adherence of *E. coli* (Bilge *et al.*, 1989); DAS100 (heat-stable enterotoxin human variant *estAh*), DAS101 (heat-stable enterotoxin, porcine variant *estAp*) and G119 (heat-labile enterotoxin *eltB*); and JPN16, the plasmid marker EPEC adherence factor (EAF) gene probe (Nataro *et al.*, 1985), MSD207 detecting the bundle-forming pilus gene (*bfpA*) (Girón *et al.*, 1993) and CVD434, *E. coli* attaching and effacing gene (*eae*) (Jerse *et al.*, 1990). Only dot-blot-positive *E. coli* strains were characterized further as described below.

Serotyping. Identification of somatic (O) and flagella (H) antigens was carried out by tube and microtitre-plate agglutination with the specific antisera O1–O181, supplemented with the presumptive new O groups OX182–OX186, and H1–H56 using methods described by Ørskov & Ørskov (1984).

Antimicrobial susceptibility testing by MIC. Antimicrobial susceptibility testing was carried out for 40 DEC and 6 *Shigella* spp. using Sensititre (TREK Diagnostic Systems), a commercially available MIC technique using dehydrated antimicrobials in microtitre wells. The wells were inoculated and incubated according to the Clinical and Laboratory Standards Institute (CLSI) (formerly the National Committee for Clinical Laboratory Standards) (NCCLS, 1997). The MIC was defined as the lowest concentration of antimicrobial with no visible bacterial growth and the breakpoints used are shown in Table 2. *E. coli* ATCC 25922 was used for quality control and the MIC values for the strains were evaluated in accordance with CLSI guidelines.

PFGE. The PulseNet method of the Centers for Disease Control and Prevention (PulseNet USA, 2004) was used for PFGE typing of the *E. coli* isolates. *Xba*I was used for genomic DNA digestion. The fragments obtained with this restriction enzyme were resolved using a contour-clamped homogeneous electric field apparatus (CHEF-II Mapper; Bio-Rad). DNA band patterns were visualized by UV illumination, photographed, analysed and compared using GEL COMPAR II software (Applied Maths). We used the band-based dice

similarity coefficient and the UPGMA dendrogram type with a position tolerance setting of 1.5% for both optimization and band comparison.

Data analysis. Incidence rates of diarrhoea were calculated for the children cohort over the follow-up period. To estimate pathogenicity of the various agents, we estimated the odds ratio by multivariate logistic regression analyses. The analyses were adjusted for age by using an indicator variable for age groups 0–11, 12–23, and ≥24 months of age. A level of significance of 0.1 presented by a 90% confidence interval (CI) was selected. Data were analysed with STATA 8 (Stata).

Ethical considerations. The children were recruited in the study after obtaining informed consent from their parents. The parents

Table 2. Break-point values for MIC testing of different DEC types

Antimicrobial agent	Breakpoint (µg ml ⁻¹)	Test range (µg ml ⁻¹)
Ampicillin (AMP)	32	1–32
Amoxicillin/clavulanic acid (AUG2)	32	2–32
Apramycin (APR)	32	4–64
Ceftiofur (XNL)	8	0.5–8
Cephalothin (CEP)	32	2–64
Chloramphenicol (CHL)	32	2–64
Ciprofloxacin (CIP)	0.125/4	0.03–4
Colistin (COL)	16	4–64
Florfenicol (FFN)	32	2–64
Gentamicin (GEN)	8	1–32
Nalidixic acid (NAL)	32	8–128
Spectinomycin (SPE)	128	4–128
Neomycin (NEO)	16	2–32
Streptomycin (STR)	32	4–64
Sulfonamide (SMX)	512	64–1024
Tetracycline (TET)	16	2–32
Trimethoprim (TMP)	16	4–32

were free to decide whether to continue or withdraw their children from the follow-up study at any time during the study period. Medical treatment (oral rehydration solution and certain antimicrobials prescribed by the medical doctor at the communal station) was provided free of charge to any child who developed an episode of diarrhoea. Ethical clearance for the study was provided by the Medical Ethics Committee of NIHE, Hanoi.

RESULTS AND DISCUSSION

Occurrence of enteric pathogens and incidence of diarrhoea

A total of 222 children, including 31 newborns, under 72 months old (median age 27 months, mean 30 months, range 1–68 months; 54.6 % boys) from 182 households were enrolled in the 18.5 month follow-up and followed for 101 509 days. A total of 173 episodes of diarrhoea was reported during 100 743 days at risk, giving an incidence of 0.63 episodes per year at risk. The highest incidence occurred in infants of <12 months of age with 1.3 episodes per year. This is lower than morbidity rates reported from prospective studies in 20 countries published between 1990 and 2000 (Kosek *et al.*, 2003), which reported mean global estimates of 2.7 episodes per year in children aged 0–5 months and 4.8 per year in children aged 6–11 months.

A total of 111 stool specimens from cases, and the same number from controls, were analysed for enteric pathogens. We detected an enteric pathogen in 73 children (65.7 %) with diarrhoea, compared with 41 controls (36.9 %) ($P<0.0001$, Table 3). This is higher than some studies where enteric pathogens were found in 46 (el Sheikh & el Assouli, 2001; El Mohamady *et al.*, 2006), 50 (Mølbak *et al.*, 1994) and 54 %

(Olesen *et al.*, 2005) of cases, and 22 % (Olesen *et al.*, 2005) and 28 % (Ogunsanya *et al.*, 1994) of controls. The findings were similar to other studies (Youssef *et al.*, 2000; Vu Nguyen *et al.*, 2006), including a multi-centre study in five countries (Huilan *et al.*, 1991), but lower than studies in Bangladesh (75 % of cases and 44 % of controls) (Albert *et al.*, 1999), Jordan (78 % of cases) (Nimri & Meqdam, 2004), Guinea-Bissau (48 % of controls) (Mølbak *et al.*, 1994) and Tanzania (52 % of controls) (Gascon *et al.*, 2000). The majority of these other studies were based on findings from patients seeking medical attention with a general practitioner (Olesen *et al.*, 2005) or at clinics (Ogunsanya *et al.*, 1994; Albert *et al.*, 1999; Gascon *et al.*, 2000), outpatient facilities (el Sheikh & el Assouli, 2001; Nimri & Meqdam, 2004; El Mohamady *et al.*, 2006; Vu Nguyen *et al.*, 2006) or inpatient hospitals (Youssef *et al.*, 2000). Only a few were community based as in this study. In Guinea-Bissau, a potential enteropathogen was found in 50 % of 1219 diarrhoeal episodes and 48 % of 511 asymptomatic controls in a 1 year community study of childhood diarrhoea (Mølbak *et al.*, 1994), and in Bangladesh 58 % of stools from children with persistent diarrhoea, 60 % from children with acute diarrhoea and 56 % from healthy controls were positive for enteropathogens (Baqui *et al.*, 1992). These different studies are not all directly comparable, as the microbiological methods were different and the range of pathogens studied varied. One limitation of the present study was the restricted range of gastrointestinal parasites studied (e.g. we did not examine for *Cryptosporidium* spp.); however, we did include a detailed diagnostic battery for DEC.

Fig. 1 shows the relative monthly prevalence of DEC, *Entamoeba histolytica* and rotavirus detected in the study in stools from both cases and controls during the 18.5 month

Table 3. Detection of enteric pathogens (DEC genes by multiplex PCR) in stool samples from children with and without diarrhoea living in Yen So commune in peri-urban Hanoi

Pathogen	No. positive (%) [no. of DEC strains isolated for characterization]		Odds ratio (95 % CI)*	P value†
	Cases (n=111)	Controls (n=111)		
Rotavirus	19 (17.1)	5 (4.5)	4.5 (1.6–13.0)	0.006
<i>Entamoeba histolytica</i>	17 (15.3)	5 (4.5)	4.4 (1.8–10.8)	0.006
DEC	25 (22.5) [22]	26 (23.4) [18]	0.9 (0.4–1.8)	0.71
EAggEC	11 [9]	15 [12]	0.4 (0.1–1.0)	0.051
A/EEC and EPEC	7 [7]	5 [5]	2.7 (0.7–10.4)	0.1
ETEC	4 [3]	5 [1]	1.2 (0.3–5.4)	0.79
EIEC	3 [3]	1 [0]	2.6 (0.4–16.8)	0.3
<i>Shigella</i> spp.	6 (5.4)	0	NA	
Non-typhoid <i>Salmonella</i>	4 (3.6)	3 (2.7)	1.0 (0.2–6.3)	0.98
<i>Campylobacter jejuni</i>	2	2	1.4 (0.1–14.6)	0.76
Total	73 (65.7)	41 (36.9)	3.55 (1.97–6.42)	0.0005

NA, Not applicable.

*Odd ratios adjusted for gender and age groups (0–23 and ≥ 24 months); the reference age group was the children older than 24 months of age.

†Bold indicates a significant difference ($P<0.005$).

Fig. 1. Relative monthly prevalence of enteropathogens detected in stools from 222 children during an 18.5 month follow-up period in Yen So commune, Hanoi, Vietnam, 2002–2004. Results for September and October are not shown. ◆, DEC; ■, rotavirus; ●, *E. histolytica*.

follow-up period. The mean number of stools tested per month (except for September and October) was 22 (range 6–51 stools). Rotavirus prevalence was highest during the winter months from October to February, where its seasonal trend was similar to other studies (Nguyen *et al.*, 2004; Van Man *et al.*, 2005). From May to December, DEC and *Entamoeba histolytica* were detected, with no obvious seasonality. Monthly prevalences of *Shigella* spp., *Salmonella* spp. and *Campylobacter* spp. are not shown in the figure and were distributed sparsely throughout the year.

In order of observed frequency, rotaviruses, *Entamoeba histolytica*, and *Shigella* spp. were found at higher prevalences in cases than in controls ($P < 0.05$, Table 3). The six cases of *Shigella* infection included one with *Shigella dysenteriae*, one double infection with *Shigella sonnei* and *S. dysenteriae*, and *S. sonnei* was isolated from the remaining four children. As in other areas of the world, rotavirus is a major cause of diarrhoeal illness, characterized by vomiting and watery diarrhoea. Most of the rotavirus cases were in children less than 3 years of age (15/19 cases). The findings in our study are in concordance with other studies in Vietnam (Nguyen *et al.*, 2001, 2004; Van Man *et al.*, 2005), Thailand (Echeverria *et al.*, 1989, 1994), Denmark (Olesen *et al.*, 2005) and Jordan (Youssef *et al.*, 2000), but not as high as in children less than 12 years in Libya (26.6%) (Ali *et al.*, 2005). Among the parasites, *Entamoeba histolytica*/*Entamoeba dispar* was surprisingly common (15.3% of cases and 4.5% of controls) and was associated with diarrhoea (odds ratio of 4.38). This was much higher than the observed 1.8% from children with acute diarrhoea in Bangladesh (Baqui *et al.*, 1992). A high prevalence of the *Entamoeba histolytica*/*Entamoeba dispar* complex (22%) has been reported from children of less than 14 years in Venezuela (Diaz *et al.*, 2006), and as 11.8% in children less

than 12 years in Libya (Ali *et al.*, 2005). In both studies, increasing age was associated with infection. One limitation of these studies, including our own, however, was that they did not differentiate between *Entamoeba histolytica* and *Entamoeba dispar*. This differentiation would be highly relevant in future studies attempting to reassess the epidemiology and transmission of amoebiasis and *Entamoeba histolytica* in particular, which has been shown to be negatively associated with the growth of pre-school children in Dhaka, Bangladesh (Mondal *et al.*, 2006), and an unusually high incidence of liver abscess in adults in central Vietnam (Blessmann *et al.*, 2002).

Among the bacterial pathogens, *Shigella* and EIEC, which share clinical and epidemiological features, was the most important group, accounting for 8% of cases of diarrhoea. This is a relatively high prevalence bearing in mind that the study was community-based where mild cases tend to dominate, and was similar to other findings (Albert *et al.*, 1999; Youssef *et al.*, 2000; Orlandi *et al.*, 2006; Vu Nguyen *et al.*, 2006), but definitely lower than in Libya (Ali *et al.*, 2005). *Shigella* and EIEC are non-zoonotic bacteria that are transmitted primarily by person-to-person spread, but part of the reason for the high prevalence could also be due to exposure to wastewater contaminated with human faeces. Clinically, the infection was characterized by fever and abdominal pain. Other DEC were also commonly found, but at high rates among both cases and controls. Unexpectedly, ETEC did not have a dominating role. VTEC, *Vibrio* spp., *G. lamblia* and *Cyclospora* spp. were not isolated from any specimen. Seventeen cases (15.3%) were infected with more than one enteric pathogen.

Fig. 2. Multiplex PCR amplification of DEC reference strains from pure cultures. Lanes: 1, VTEC ATCC 43889 (*eae*, *vtx2* and *vtx1*); 2, EAggEC (*aatA*); 3, EPEC ATCC 43887 (*eae* and *bfpA*); 4, ETEC ATCC 35401 (*eltB* and *estB*); 5, EIEC ATCC 43893 (*ipaH*); 6, VTEC 43890 (*eae* and *vtx1*); 7, sample D2842 (*ipaH*); 8, negative control ATCC 11775; M, DNA marker, with fragment sizes indicated (bp).

Characterization of DEC

Fig. 2 shows the band patterns using multiplex PCR obtained with reference strains from pure cultures. Multiplex PCR with the pooled DNA (five to seven colonies from each faecal sample) from a total of 222 faecal samples yielded amplicons for 51 specimens (Table 3). In multiplex PCRs, 11 pools of five to seven colonies from each sample were positive, but isolation of single viable cultures was unsuccessful from 3 cases (two EAggEC and one ETEC) and 8 controls (three EAggEC, four ETEC and

one EIEC). Eleven samples therefore could not be tested by dot-blot hybridization. Dot-blot hybridization results of the 40 PCR-positive strains were in accordance with the PCR results. If isolation was unsuccessful, samples were still considered to be positive because of the high sensitivity of the PCR assay (Svenungsson *et al.*, 2000). This was expected, in view of the high sensitivity of PCR in comparison with conventional culture procedures. The presence of other enterotoxin-producing bacteria (e.g. *Klebsiella pneumoniae* and *Citrobacter freundii*) could not be excluded, but results from previous studies indicate that

Table 4. Pathogenic group, virulence genes, serotypes and PFGE types of 40 *E. coli* strains isolated from cases of diarrhoea and controls

Number	Pathogenic group	Virulence gene	Serotype	PFGE pattern	Status
D2858	A/EEC	<i>eae</i>	O13:NM	NT	Control
D2739	A/EEC	<i>eae</i>	O156:NM	16	Control
D2740	A/EEC	<i>eae</i>	O177:NM	18	Control
D2990	A/EEC	<i>eae</i>	O rough:H19	11	Control
D2743	A/EEC	<i>eae</i> , <i>bfpA</i>	O49:H10	7	Control
D2725	A/EEC	<i>eae</i>	O158:H19	34	Diarrhoea
D2729	A/EEC	<i>eae</i>	O rough:NM	28	Diarrhoea
D2734	A/EEC	<i>eae</i>	O3:H19	19	Diarrhoea
D2736	A/EEC	<i>eae</i>	O49:H46	23	Diarrhoea
D2738	EPEC	<i>eae</i>	O157:NM	33	Diarrhoea
D2741	EPEC*	<i>eae</i> , <i>bfpA</i>	O119:NM	30	Diarrhoea
D2730	EPEC*	<i>eae</i> , EAF	O111:H9	29	Diarrhoea
D2755	EAggEC	<i>aatA</i>	O86:H30	9	Control
D2764	EAggEC	<i>aatA</i>	O59:NM	8	Control
D2766	EAggEC	<i>aatA</i>	O+:H10	12	Control
D2773	EAggEC	<i>aatA</i>	O117:H37	13	Control
D2778	EAggEC	<i>aatA</i>	O rough:H30	10	Control
D2780	EAggEC	<i>aatA</i>	O6:H10	2	Control
D2781	EAggEC	<i>aatA</i>	O145:H4	15	Control
D2768	EAggEC	<i>aatA</i> , <i>astA</i>	O14:NM	5	Control
D2779	EAggEC	<i>aatA</i> , <i>astA</i>	O134:H27	NT	Control
D2784	EAggEC	<i>aatA</i> , <i>astA</i>	O15:NM	6	Control
D2758	EAggEC	<i>aatA</i> , <i>astA</i> , EAF	O13:H30	4	Control
D2762	EAggEC	<i>aatA</i> , EAF	O171:NM	17	Control
D2761	EAggEC	<i>aatA</i>	O171:H7	36	Diarrhoea
D2769	EAggEC	<i>aatA</i>	O rough:H31	25	Diarrhoea
D2775	EAggEC	<i>aatA</i>	O14:H27	20	Diarrhoea
D2776	EAggEC	<i>aatA</i>	O128abc:H12	31	Diarrhoea
D2772	EAggEC	<i>aatA</i>	O rough:H10	26	Diarrhoea
D2759	EAggEC	<i>aatA</i> , <i>astA</i>	O rough:H10	24	Diarrhoea
D2756	EAggEC	<i>aatA</i> , <i>astA</i>	O15:NM	21	Diarrhoea
D2767	EAggEC	<i>aatA</i> , <i>astA</i>	O143:H3	32	Diarrhoea
D2771	EAggEC	<i>aatA</i> , <i>astA</i>	O176:H34	37	Diarrhoea
D2816	EIEC	EIEC, <i>ipaH</i>	O rough:NM	27	Diarrhoea
D2818	EIEC	EIEC, <i>ipaH</i>	O164:NM	35	Diarrhoea
D2842	EIEC	<i>ipaH</i>	OX186:NM	38	Diarrhoea
D2677	ETEC	<i>eltB</i> , <i>estAh</i>	O6:H16	1	Control
D2671	ETEC	<i>eltB</i>	O15:H51	22	Diarrhoea
D2676	ETEC	<i>eltB</i> , <i>estAh</i>	O6:H16	1	Diarrhoea
D2673	ETEC	<i>estAh</i>	O6:H16	1	Diarrhoea

NM, Non-motile; NT, not typable by PFGE (the gel was smeared); O rough, autoagglutinable; O+, not typable.

*Two serotypes belonging to classical typical EPEC serotypes (Trabulsi *et al.*, 2002).

most enterotoxin-producing strains isolated from clinical samples are *E. coli* (Jertborn & Svennerholm, 1991; Nataro & Kaper, 1998).

Pathogenic group, virulence genes, serotype and PFGE patterns of isolates from cases and controls are shown in Table 4. Two strains were not typable by PFGE and resulted in smears on the gels in repeated testing. A total of 20 serotypes was isolated from 22 cases and 18 controls. EIEC strains were only isolated from cases of diarrhoea, one of which had a presumptive new O antigen (OX186), currently under investigation. O15:NM *aatA* and *astA* were isolated from one case and one control. Two O rough:H10 strains – one *aatA* and *astA*, one *aatA* – were isolated from cases. O171:NM *aatA* and EAF was isolated

from one control and O171:H7 *aatA* of less than 75% similarity by PFGE was isolated from one case (Fig. 3). Two cases and one control of O6:H16 were 95% similar by PFGE (Fig. 3). Only two *eae*-positive strains belonged to the classical typical EPEC serotypes, O111:H9 and O119:NM (Trabulsi *et al.*, 2002), with the presence of EAF or *bfpA*, and were from cases of diarrhoea. A strain of O157:NM from diarrhoea could represent one of the newly described EPEC serotypes (Scotland *et al.*, 1992; Makino *et al.*, 1999). Of particular interest was the finding that the plasmid gene *aatA* – thought to be a specific marker for the most virulent typical EAggEC strains – was found together with the EPEC plasmid marker EAF in two strains from controls. The significance of this finding is unclear, but illustrates the difficulties in categorizing DEC. Neither

Fig. 3. Dendrogram of 38 *Escherichia coli* strains based on PFGE typing patterns produced using the band-based dice similarity coefficient and UPGMA. D, Diarrhoea; C, control.

serotyping nor PFGE revealed any significant similarities between isolates from either cases or controls. Similarly, no differences were observed among isolates from cases and controls. Strains of the same serotype from cases and controls were low in similarity (67 %) by PFGE (Fig. 3). However, three EAggEC strains (O+ :H10 from one control and two O rough:H10 from cases) were 90 % similar and two EPEC (O177:NM and O119:NM) were closely related with 89 % similarity in their PFGE patterns. Such trends have been observed previously among EPEC, ETEC and EAggEC strains in India (Kahali *et al.*, 2004).

Antimicrobial susceptibility testing

The overall results from the antimicrobial susceptibility testing for DEC are shown in Fig. 4. The trend of resistance was different among DEC types. One ETEC strain was multi-resistant to NAL, CHL, SMX, STR, TET and TMP, with the remaining three ETEC strains found to be sensitive to all antimicrobials tested. Resistance to AMP, SMX, STR, TET and TMP was shown by 71–86 % of EAggEC. Six *eae* positives (including three EPEC strains) were resistant to AMP, SMX and TMP; four *eae* positives (including three EPEC strains) and five *eae* positives (including three EPEC strains) were resistant to TET and STR, of which two strains were multi-resistant to seven antimicrobials including NAL and GEN. One out of three EIEC strains was sensitive to all antimicrobials, whilst the two remaining EIEC strains were resistant to AMP, SMX, STR and TMP, and showed intermediate resistance to CEP. All *Shigella* strains were resistant to SMX, SPE, STR, TET and TMP. These patterns of multi-resistance have been observed by others (Anh *et al.*, 2001; Nguyen *et al.*, 2005b). Testing for all antimicrobials commonly used for diarrhoea treatment (SMX, TMP, TET, SPE and STR) resulted in resistance values twice as

high as the breakpoint values (>1024 , >32 , >32 , >256 and $>64 \mu\text{g ml}^{-1}$, respectively), which is in accordance with other studies, and showed a low activity of these antimicrobials against DEC and *Shigella* strains (Nguyen *et al.*, 2005b). Therefore, local information about antimicrobial resistance should be used in clinical management, and treatment guidelines should be updated.

Conclusions

Our further characterization of DEC strains showed that their serotypes were highly heterogeneous and were not typical of any of the known DEC pathotypes except for two typical EPEC strains from children with diarrhoea. From the results of serogroups, virulence genes, antimicrobial profiles 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 features analysed in this study. To our knowledge, this is first report regarding clonal analysis employing a molecular approach on DEC strains from cases of diarrhoea in Vietnam. From the observed set of strains, it could be inferred that the DEC strains exhibited a high degree of heterogeneity in their genetic make-up. However, prospective molecular epidemiological studies in several locations are required before arriving at any conclusion. These studies are ongoing.

The present study was conducted in an area where a large part of the adult population is exposed to untreated wastewater as part of agricultural activities and fishpond culture. Although conducted with a small number of samples, this study provides a valuable indication of childhood diarrhoeal disease morbidity and aetiology in an understudied area (risk of diarrhoea in a wastewater-use area) and part of the world (Vietnam). However, the incidence of diarrhoea was lower than in many other developing countries (0.63 episodes per year), and the overall detection and prevalence of enteric pathogens occurred at roughly similar levels. Thus, living in a wastewater-use area may not have a major influence on the incidence of childhood diarrhoea or on the overall detection and prevalence of enteric pathogens. A more detailed analytical epidemiological study is required to address this aspect further. The study area was located near Hanoi, where drinking water, sanitation and food safety are relatively well developed, and access to health care is better than in other rural areas. This may have contributed to the relatively low diarrhoeal morbidity in the population. Thus, the relative contribution of different pathogens accounting for diarrhoea may vary depending on the specific area. With regard to local intervention, treatment approaches and vaccine development strategies, as well as further studies on aetiology and characterization, are required. This study suggests that, if vaccines for rotavirus and *Shigella*/EIEC become available as part of a routine schedule for childhood immunization, they could have a major impact on the incidence of diarrhoea and improvement of child health.

Fig. 4. Antimicrobial susceptibilities of DEC strains. For antimicrobial agent abbreviations see Table 2. Grey bars, sensitive; black bars, resistant; white bars, intermediate.

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