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Shiga toxin-producing *Escherichia coli* isolated from patients with diarrhoea in Bangladesh

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The prevalence of Shiga toxin-producing *Escherichia coli* (STEC) and its characteristics were determined among hospitalized patients with diarrhoea and children with diarrhoea in an urban slum community of Dhaka city using sensitive culture and PCR methods. Stool samples were collected from 410 patients with diarrhoea enrolled in the 2 % surveillance system (every 50th patient attending the hospital with diarrhoeal disease is included) at the ICDDR,B hospital and from 160 children of 2–5 years of age with diarrhoea living in an urban slum in Dhaka, between September 2004 and April 2005. Shiga toxin genes (*stx*) were detected by multiplex PCR in the enrichment broth of nine samples (2.2 %) from hospitalized patients and 11 samples (6.9 %) from the community patients. STEC was isolated from five stool samples with positive PCR results using a colony patch technique. All five isolates were positive in the Vero cell assay and PCR fragments of *stx* genes were confirmed by sequencing. Two isolates were positive for the *E. coli* attaching-and-effacing (*eae*) gene and four were positive for the enterohaemolysin (hly_{EHEC}) gene and enterohaemolysin production. The five isolates belonged to five different serotypes: O32 : H25, O2 : H45, O76 : H19, ONT : H25 and ONT : H19. It can be concluded that STEC is not a common pathogen in Bangladesh among hospitalized patients with diarrhoea nor among mild cases of diarrhoea in the community.

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INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is an important causative agent of haemorrhagic colitis and diarrhoea-associated haemolytic–uraemic syndrome (HUS) with or without neurological complications (Karmali, 1989; Nataro & Kaper, 1998; Paton & Paton, 1998a). STEC is a serologically diverse group of foodborne, zoonotic pathogens. Serotype O157 : H7 has been the predominant type worldwide (Tarr *et al.*, 2005). However, altogether more than 200 STEC serotypes have been reported and more than 100 have been linked with human infection (Eklund *et al.*, 2001). In some geographic areas, STEC non-O157 is more commonly isolated from persons with diarrhoea or HUS than STEC O157 strains (Pradel *et al.*, 2000).

Although most sporadic cases and outbreaks have been reported from developed countries, human infections associated with STEC strains have also been described in Latin America, India and other developing countries (Kaddu-Mulindw *et al.*, 2001; Leelaporn *et al.*, 2003). In Bangladesh, the predominant group of *E. coli* associated with childhood diarrhoea is enterotoxigenic *E. coli*, accounting for approximately 20 % of all diarrhoeal cases (Qadri *et al.*, 2005). The burden of STEC-associated diarrhoea in Bangladesh is unknown. In the present study, we investigated the prevalence of STEC among hospitalized patients with diarrhoea as well as among patients with diarrhoea attending the community clinics. The STEC isolates were characterized in detail.

Abbreviation: STEC, Shiga toxin-producing *Escherichia coli*.

METHODS

Stool samples. Between September 2004 and April 2005, stool samples were collected from patients with diarrhoea enrolled in an ongoing active surveillance system at the Dhaka treatment centre operated by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). In this surveillance system, every

50th patient attending the hospital with diarrhoeal disease is included. A detailed history of the patients is obtained, including information on the age, sex and clinical presentation. Stool samples were collected in sterile McCartney bottles and transported to the laboratory within 1 h.

In the same period, stool samples were collected from children having complaints of diarrhoea attending the Centre's community clinic at Mirpur, an urban slum in Dhaka. The inhabitants of Mirpur are of Bihari ethnic origin and settled there after the separation of Bangladesh from Pakistan in 1971. The area is densely populated and has poor sanitary and hygiene conditions. Clinical information was collected by health care workers who visited the children and their parents every other day. Stool samples were kept on ice, and transported to the laboratory for processing within 2–4 h after collection.

Isolation procedure for STEC O157. A loopful of stool sample was inoculated into 3 ml modified tryptone soy broth (Oxoid) and incubated overnight at 37 °C. Immunomagnetic separation using Dynabeads anti-*E. coli* O157 (Dynal) was performed with 1 ml broth culture, following the manufacturer's instructions. The immunoconcentrated samples were inoculated onto two selective isolation media: sorbitol-MacConkey agar (Oxoid) supplemented with cefixime (0.05 mg l^{-1}) and tellurite (2.5 mg l^{-1}) (Oxoid) (CT-SMAC); and CHROMagar O157 (ITK Diagnostics BV) with cefixime (0.025 mg l^{-1}) and tellurite (1.25 mg l^{-1}) (½CT-CHROM). The agar plates were incubated for 18–24 h at 37 °C. Typical colonies (colourless colonies on CT-SMAC and mauve on ½CT-CHROMagar), up to 12 per plate, were selected and streaked onto tryptone soy agar (TSA) plates. The colonies on TSA plates were tested for agglutination with an *E. coli* O157 latex test kit (Murex) and a PCR for the *rfb*E_{O157} gene, which is specific for *E. coli* O157 (Table 1).

Detection and isolation of STEC non-O157 types. The overnight incubated enrichment broth was also examined by PCR using *stx*$_1$ and *stx*$_2$ primers (Table 1). DNA was extracted from 1 ml broth culture by thermal cell lysis using Chelex-100 resin (Bio-Rad) (Malorny *et al.*, 2003) and 5 µl of the DNA extract was used in the PCR. Touchdown multiplex PCR for *stx*$_1$ and *stx*$_2$ was carried out in a PTC-200 peltier thermal cycler (Bio-Rad). After initial incubation at 94 °C for 5 min, a 40-cycle amplification protocol was implemented as follows: 94 °C for 30 s, 64 °C for 30 s and 72 °C for 60 s for two cycles followed by eight cycles with decreasing annealing temperatures of 2 °C in every two cycles. When the annealing temperature of 54 °C was reached at cycle 10, the PCR was continued with these cycling parameters followed by a final extension of 10 min at 72 °C. All PCR fragments for *stx*$_1$ and *stx*$_2$ genes were subjected to sequencing. After electrophoresis, bands of PCR products were extracted and purified using the PCR Product Purification kit (Roche). Subsequently, a cycle-sequence reaction was performed using a kit (BigDye Terminator v3.1 cycle-sequencing kit; Applied Biosystems) according to the manufacturer's protocol. Purified PCR products were sequenced on an automated sequencer (ABI Prism 3100-Avant Genetic Analyser; Applied Biosystems). The chromatogram sequencing files were inspected using Chromas 2.23, and contigs were prepared using SeqMan II (DNASTAR). Nucleotide sequence similarity searches were performed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) server on the GenBank database, release 138.0.

Broth cultures that yielded positive PCR results for *stx*$_1$ and *stx*$_2$ or both were streaked onto tryptone bile X-glucuronide (TBX) agar (Oxoid) supplemented with cefixime (20 µg l^{-1}), cefsulodin (3 mg l^{-1}) and vancomycin (30 mg l^{-1}) (CCV-TBX). Single colonies including different morphological types were transferred from the CCV-TBX and plated onto Luria agar (Difco) to create a grid pattern of 96 colonies (12 × 8). The plates were incubated overnight at 37 °C. The isolates were subjected to PCR for the detection of *stx*$_1$ and *stx*$_2$ as described above. The number of PCR reactions was reduced to 20 by pooling the colonies per row and per column in 500 µl sterile distilled water. DNA was extracted by boiling the suspensions for 10 min and 5 µl of the supernatant was used in the PCR. The isolates with positive PCR results were identified as *E. coli* by an API 20E test (bioMérieux).

Characterization of STEC isolates. All isolates were sent to the National Institute of Public Health and the Environment (RIVM) in Bilthoven, the Netherlands, for O and H typing. Production of Shiga toxin was determined by Vero cell culture assay (Karmali *et al.*, 1985a). The isolates were tested by PCR for the presence of the *E. coli* attaching-and-effacing (*eae*) and the enterohaemolysin (*hly*$_{EHEC}$) genes (Heuvelink *et al.*, 1995; Wieler *et al.*, 1996). The oligonucleotide primers used for PCR are listed in Table 1. Enterohaemolytic activity was examined by streaking the isolates onto tryptone soy agar supplemented with 10 mM $CaCl_2$ and 5 % defibrinated sheep blood cells washed three times in PBS (pH 7.2), as described previously (Beutin *et al.*, 1989). The plates were observed for haemolysis after 4 h (α-haemolysis) and after overnight incubation in ambient air at 37 °C (enterohaemolysis or nonhaemolysis).

Isolation of other enteric pathogens. All samples from hospitalized patients were examined for other enteric pathogens in addition to STEC, including *Vibrio cholerae*, *Salmonella* spp., *Shigella* spp. and *Aeromonas* spp., using standard microbiology procedures (World Health Organization, 1987).

Statistical analysis. Data were entered and statistical analyses were performed using SPSS and Epi-Info. The significance of difference in

Table 1. PCR primers used in this study

Primer	Sequence (5′–3′)	Target	PCR product (bp)	Reference
stx$_1$F	CAC AAT CAG GCG TCG CCA GCG CAC TTG CT	*stx*$_1$	606	Heuvelink *et al.* (1994)
stx$_1$R	TGT TGC AGG GAT CAG TCG TAC GGG GAT GC			
stx$_2$F	CCA CAT CGG TGT CTG TTA TTA ACC ACA CC	*stx*$_2$	372	Heuvelink *et al.* (1995)
stx$_2$R	GCA GAA CTG CTC TGG ATG CAT CTC TGG TC			
*eae*F	TGCGGCACAACAGGCGGCGA	*eae*	629	Heuvelink *et al.* (1995)
*eae*R	CGGTCGCCGCACCAGGATTC			
hly$_{EHEC}$F	GAGCGAGCTAAGCAGCTTG	*hly*$_{EHEC}$	889	Wieler *et al.* (1996)
hly$_{EHEC}$R	CCTGCTCCAGAATAAACCACA			
*rfb*O157F	CGGACATCCATGTGATATGG	*rfb*E_{O157}	259	Paton & Paton (1998b)
*rfb*O157R	TTGCCTATGTACAGCTAATCC			

proportions was evaluated by the χ^2 test, and Fisher's exact test was applied when appropriate. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Over the years, STEC has been found to be associated with all forms of diarrhoeal infection ranging from watery to severe bloody diarrhoea. Bangladesh is an endemic zone for diarrhoeal diseases: every year, more than 5 % of deaths of children below 5 years of age are attributed to diarrhoea (Arifeen *et al.*, 2005). In Bangladesh, no STEC infection among the patients with diarrhoea has been reported (Albert *et al.*, 1995). The reasons might be (1) the lack of proper surveillance for STEC, (2) STEC is not present or (3) STEC is present but relatively few infections occur due to acquired immunity in the population. A study on the prevalence of STEC in neighbouring Calcutta, India, showed a very low prevalence of STEC among hospitalized patients with diarrhoea (1.4 % and 0.6 % of bloody and watery stool samples, respectively) (Khan *et al.*, 2002). The present study was aimed at measuring the burden or occurrence of STEC infection among the patients with diarrhoea in the hospital and in the community using sensitive culture and PCR methods.

Prevalence of STEC

Of 410 stool samples collected from hospitalized patients, nine (2.2 %) were positive by PCR: four for stx_2 only, three for stx_1 only and two for both stx_1 and stx_2 (Table 2). No statistically significant correlation between the age of patients and the presence of *stx* genes was observed. All *stx* positive patients were clinically diagnosed as having uncomplicated diarrhoea. All patients had watery diarrhoea and mucus was present in the stool samples. Vomiting was recorded for seven patients (78 %). Interestingly, seven of the nine patients were primarily diagnosed with a *V. cholerae* O1 infection (Table 2). The relative frequency of other enteric pathogens detected in the hospital samples is shown

Table 2. Presence of *stx* genes in stool samples, clinical manifestation and stool characteristics of patients positive for *stx* genes

stx, Shiga toxin; F, female; M, male; Metro, metronidazole; UID, unidentified; Cotri, cotrimoxazole; Ery, erythromycin; UCD, uncomplicated diarrhoea; ND, not done.

Patient code	Presence of *stx* genes		Patient history								Stool characteristics			
	stx_1	stx_2	Age (years)	Sex	Fever	Vomiting	Abdominal pain	Convulsion	Chemotherapy before arrival	Diagnosis	Watery	Bloody	Mucus	Presence of other pathogen in stool
Hospitalized patients														
Am 3	+	−	70	F	−	<10 times	−	−	Metro	UCD	+	−	++	None
Am 35	−	+	7	M	−	<10 times	−	−	None	UCD	+	−	+	*V. cholerae* O1
Am 84	+	+	22	F	−	>10 times	+	−	Metro	UCD	+	−	+	*V. cholerae* O1
Am 107	+	+	35	M	−	<10 times	−	−	Metro	UCD	+	−	++	*V. cholerae* O1
Am 113	−	+	25	F	−	<10 times	−	−	UID	UCD	+	−	+	None
Am 167	−	+	2	F	−	−	+	−	UID	UCD	+	−	+	*V. cholerae* O1
Am 181	+	−	6	F	+	<10 times	+	−	UID	UCD	+	−	++	*V. cholerae* O1
Am 281	−	+	18	F	−	−	+	−	None	UCD	+	−	+	*V. cholerae* O1
Am 403	+	−	18	F	−	>10 times	−	−	None	UCD	+	−	+	*V. cholerae* O1
Community patients														
AI 1	+	+	10	F	+	−	+	−	Cotri	UCD	+	−	−	ND
AI 3	+	+	7	M	−	−	+	−	None	UCD	+	−	−	ND
AI 9	+	−	10	M	−	−	+	−	Ery	UCD	+	−	−	ND
AI 11	+	−	10	F	−	−	+	−	None	UCD	+	−	−	ND
AI 25	+	−	11	F	−	−	−	−	None	UCD	+	−	+	ND
AI 48	+	+	8	F	−	−	−	−	None	UCD	+	−	−	ND
AI 101	−	+	8	F	+	−	+	−	None	UCD	+	−	−	ND
AI 103	−	+	8	F	+	−	−	−	None	UCD	+	−	−	ND
AI 130	+	−	10	M	+	−	+	−	None	UCD	+	−	−	ND
AI 132	−	+	11	M	−	−	−	−	None	UCD	+	−	−	ND
AI 160	+	−	10	M	−	−	+	−	None	UCD	+	−	−	ND

in Table 3. Of 160 samples collected from the community patients with diarrhoea, 11 (6.9 %) were found to be positive: five for stx_1, three for stx_2 and three for both stx_1 and stx_2 (Table 2). All patients had mild diarrhoea and were diagnosed as having uncomplicated diarrhoea. STEC could be isolated from two (22 %) among nine PCR positive samples from the hospitalized patients and three (27 %) of 11 PCR positive samples from community patients. The prevalence of STEC in hospitalized patients with diarrhoea was very low (0.5 %), and was somewhat higher among the community patients with diarrhoea (1.9 %).

Failure to isolate the STEC non-O157 from PCR positive stool samples is still a common problem encountered by researchers around the world (Blanco *et al.*, 2004). According to previous studies, the non-isolation of viable STEC from PCR-positive samples might be due to the presence of very low numbers of bacteria (Khan *et al.*, 2002), the presence of free *stx* phages in the sample (Karmali *et al.*, 1985b) and loss of *stx* genes upon subcultivation of strains (Karch *et al.*, 1992). In routine diagnostics, there is no definitive biochemical characteristic, such as sorbitol fermentation in the case of serogroup O157, which can identify STEC irrespective of serotypes from the commensal flora. Therefore, selection of suitable culture media for isolation of STEC non-O157 serogroups is always difficult. In this study, we used TBX agar; this medium allows basic differentiation of *E. coli* colonies but does not discriminate between STEC and non-STEC. In order to make it more selective we supplemented TBX agar with cefixime (20 μg l^{-1}), cefsulodin (3 mg l^{-1}) and vancomycin (30 mg l^{-1}). Antibiotics at these concentrations were used previously with blood agar in order to isolate STEC non-O157 serogroups (Lehmacher *et al.*, 1998). These antibiotic supplements suppress the growth of Gram-positive bacteria, *Proteus* spp. and *Pseudomonas* spp., and other *E. coli*.

Table 3. Prevalence of other bacterial enteropathogens in stool samples from hospitalized patients examined for Shiga toxin-producing *E. coli*

Enteropathogen	Prevalence (%)
Vibrio cholerae O1 El Tor Inaba	19.0
O1 El Tor Ogawa	18.0
Shigella flexneri	2.2
Shigella boydii	0.7
Shigella dysenteriae	0.2
Salmonella Typhi	0.2
Salmonella paratyphi B	0.2
Salmonella Group C1	0.7
Salmonella Group D	0.2
Salmonella Group C2	0.2
STEC O157	0.0
STEC non-O157	0.5

Characterization of STEC isolates

No STEC O157 was isolated from patients in this study. The serotypes of STEC isolates were different: O32 : H25, O2 : H45, O76 : H19, ONT : H25, ONT : H19. The characteristics of the O76 : H19 isolate in the present study were similar to those described previously (Stephan & Untermann, 1999) for O76 : H19 isolates from asymptomatic human carriers, except for the stx_2 gene, which was additionally present in our isolate. STEC ONT : H25 was previously isolated from healthy cattle in Canada with a higher prevalence (22.5 %) compared to the prevalence of *E. coli* O157 : H7 (15 %). The virulence properties of our isolate and the isolates from Canada were similar except for the type of *stx* gene. In contrast to the isolate in the study, which was positive for the stx_1, all isolates from Canada were positive for stx_2 (Sheng *et al.*, 2005). One isolate from the hospitalized patient was of serotype O32 : H25. This serotype has not previously been described as being associated with STEC infection in humans or isolated from animals. The other two serotypes in the current study, O2 : H45 and ONT : H19, were previously isolated from cattle in Hong Kong (Leung *et al.*, 2003) and India (Pal *et al.*, 1999), respectively.

All five isolates were positive in the Vero cell cytotoxicity test. One from a hospitalized patient and one from a community patient were positive for the *eae* gene (Table 4). All three isolates from the community patients and one isolate from the hospitalized patients were positive for the hly_{EHEC} gene (Table 4). It has been suggested in a previous study that there is an association among the locus of enterocyte effacement (i.e. the location of the *eae* gene), the enterohaemorrhagic *E. coli* haemolysin plasmid, and the haemolysin itself of STEC non-O157 isolates (Boerling *et al.*, 1998). *E. coli* possessing the *eae* gene were statistically more likely to be enterohaemolytic than *E. coli* that did not carry this gene (Eklund *et al.*, 2001). STEC having both the *eae*

Table 4. Shiga toxin-producing *E. coli* isolated from patients with diarrhoea in Bangladesh

No. of strain	Source*	Serotype	PCR result†	Ehly‡
Am 181	A	O32 : H25	stx_1+, *eae*−, hly_{EHEC}−	−
Am 281	A	O2 : H45	stx_2+, *eae*+, hly_{EHEC}+	+
AI 3	B	O76 : H19	$stx_{1,2}$+, *eae*−, hly_{EHEC}+	+
AI 130	B	ONT : H25	stx_1+, *eae*+, hly_{EHEC}+	+
AI 132	B	ONT : H19	stx_2+, *eae*−, hly_{EHEC}+	+

*Samples were collected from two different groups of patients with diarrhoea: A, hospitalized patients; B, community patients.

†Carriage of stx_1 or stx_2 or *eae* or hly_{EHEC} gene. +, Positive; −, negative.

‡Ehly, Enterohaemolysin production: +, enterohaemolysin positive; −, no haemolysis or α-haemolysis.

gene and haemolytic properties (hly_{EHEC} gene) is more virulent and causes more human infection than strains carrying only the *stx* genes (Eklund *et al.*, 2001; Oswald *et al.*, 2000; Schmidt *et al.*, 1995). However, we did not find a more severe clinical presentation in those patients from whom STEC was isolated.

Although STEC has been isolated sporadically from different regions of developing countries, it was never implicated as a major causative agent of diarrhoea. In a previous study in Bangladesh, a total of 452 children with diarrhoea and 602 matched control children without diarrhoea were investigated for the presence of diarrhoeagenic *E. coli* (Albert *et al.*, 1995). In children (up to 5 years of age) with diarrhoea, enteropathogenic *E. coli* (EPEC) was the most prevalent (15.5 %), followed by enterotoxigenic *E. coli* (12 %), enteroaggregative *E. coli* (9.5 %) and diffuse adherent *E. coli* (8.2 %). Enterohaemorrhagic *E. coli* possessing a *stx* gene was not detected in any of the children with diarrhoea but was detected in five children without diarrhoea. No further characterization of these five isolates was performed. STEC-identified diarrhoeal cases in developing countries are often infected with other pathogens as well; for example, in India, 58 % of the STEC positive patients were co-infected with other enteric pathogens (Khan *et al.*, 2002). This trend was also observed in the current study; seven of the nine PCR positive samples from hospitalized patients were also positive for *V. cholerae*. In fact, *V. cholerae* was the most common pathogen isolated from the hospitalized patients included in this study, which accounted for 37 % of samples (Table 3). Therefore, the specific role of STEC in causing diarrhoeal illness in this area is difficult to estimate. The reasons for the low prevalence of STEC-associated diarrhoea among hospitalized and community patients are still not clear. However, protective immunity against STEC could be an explanation, and was addressed by most of the studies done in developing countries (Gianantonio *et al.*, 1964; Lopez *et al.*, 1989; Navarro *et al.*, 2003; Seriwatana *et al.*, 1988). The low prevalence of STEC in Calcutta and possibly other places in India was explained by the fact that Indians acquire protective antibodies at an early age or their cooking practices effectively eliminate STEC (Khan *et al.*, 2002). According to previous studies, this immunity can be O group-specific or cross-reactive (mucosal) and is normally acquired during infancy (Lopez *et al.*, 1989; Navarro *et al.*, 2003). This could be associated with the repeated antigenic stimulation in a contaminated environment where diarrhoeal diseases are considered endemic (Navarro *et al.*, 2003). It has also been suggested (Beutin *et al.*, 1998) that EPEC infections in early childhood confer cross-reacting protective immunity against STEC types that share common antigens (such as LPS and intimin) with classical EPEC strains. However, a detailed study of the immune status of patients with diarrhoea as well as healthy controls in areas where enteric pathogens are considered to be endemic should be carried out in order to explain these phenomena.

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