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PREVALENCE AND CHARACTERISTICS OF *ESCHERICHIA COLI* ISOLATES HARBOURING SHIGA TOXIN GENES (*STX*) FROM ACUTE DIARRHOEAL PATIENTS IN DHAKA, BANGLADESH

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Abstract: Shiga toxin genes (*stx*) harbouring *Escherichia coli* (STEC) strains were isolated and identified from diarrhoeal patients visiting the Dhaka Hospital of ICDDR,B: Centre for Health and Population Research, Dhaka, Bangladesh. Of the 189 *E. coli* strains isolated from 775 diarrhoeal stool specimens, 19 harboured *stx1*, and one isolate was revealed to have amplicons for both *stx1* and *stx2* by a PCR assay. Sequence analysis of the 349-bp *stx1* from representative isolates revealed 100% homology with the sequence of *stx1* available in the GenBank. Among the *stx1* positive isolates, two harboured the *eae* but none were positive for *hlyA, katP, etpD* or *saa* genes. Fifteen of the 20 *stx* positive strains could be categorized into 13 non-O157 serogroups while 4 were untypable and one was a rough strain. Most of the STEC strains were resistant to ampicillin, cephalothin, co-trimoxazole, tetracycline, and nalidixic acid. In the Vero cell assay, all the strains were negative for expression of Shiga toxin (Stx). Randomly amplified polymorphic DNA (RAPD) PCR analysis demonstrated genetic diversity. This is one of the first reports to show the presence of STEC in diarrhoeal patients in Bangladesh.
Key Words: Diarrhoea, STEC, PCR, Shiga toxin gene, Genetic diversity

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC), a distinct class of pathogenic *E. coli,* can cause a spectrum of human illnesses ranging from asymptomatic carriage to severe bloody-diarrhoea termed haemorrhagic colitis (HC), as well as life-threatening sequels such as haemolytic uremic syndrome (HUS) which has a case fatality rate of up to 5% [1, 2]. STEC has gained immense clinical importance since being recognized in food-borne outbreaks in Oregon and Michigan in 1982 [3], and subsequently it has emerged as a major public health problem in many developed countries. The rate of morbidity and mortality due to Shiga toxin-producing *E. coli* (STEC) has highlighted the threat these organisms pose to public health. The ability of STEC to cause serious disease in humans is related to the production of one or more Shiga toxins (Stx1, Stx2 or their variants), which inhibit protein synthesis of host cells leading to death [4, 5]. Stx1, Stx2 and their variants are immunologically non-cross reactive and are encoded by alleles in the genome of temperate, lambdoid bacteriophages that remain integrated in the *E. coli* chromosome [6]. Stx1 is virtually identical to Shiga toxin produced by *Shigella dysenteriae* 1, while Stx2 has only 56% identity to Stx1 [7].

The pathogenesis of STEC is triggered by a set of genes carried in the chromosome that includes a 35.5 kb pathogenicity island, termed locus of enterocyte effacement (LEE). The characteristic attaching and effacing (A/E) lesion caused by most of the STEC depends on the activity of multiple genes in the LEE including the type III secretion system and on the initiation of signal transduction events [8]. The *eae* gene is responsible for attaching to translo-

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cated intimin receptor (Tir). Some of the STEC strains harbour a 97 kb plasmid (pO157), encoding possible additional virulence genes such as *hlyA* (EHEC-HlyA) that acts as a pore-forming cytolysin on eukaryotic cells, the bifunctional catalase peroxidase (KatP), the *etpD* gene cluster that probably encodes a type II secretion pathway, and the secreted serine protease (*EspP*) [9-13]. The *saa* gene (STEC autoagglutination adhesin) encodes a novel outer membrane protein and is carried on megaplasmid of LEE-negative STEC strains, which acts as an autoagglutinating adhesin [14].

About 250 non-O157 STEC serotypes have been reported to be associated with diarrhoea, HUS and other afflictions [15, http://www.Microbionet.com.au/frames/feature/vetc/brief01.html]. In the USA, non-O157 serotypes are detected at regular intervals but lack many important virulence genes [16]. Among STEC, the serotype O157:H7 is epidemiologically significant worldwide (http:www.who.int/emcdocuments/zoonooses/whocsraph988.c.hlml). STEC infection has posed a much greater problem to developed countries than to developing countries. The low incidence of STEC in developing countries might be attributable to the complexity of recognising emerging variants [17, 18]. Though STEC has been isolated from humans and cattle in India [19], less is known about STEC in Bangladesh. An earlier study observed the presence of SLT-I and SLT-II positive *E. coli* isolates in the paediatric population in Bangladesh [20]. This study was conducted to determine the significance of STEC- related diarrhoea in the Bangladeshi setting. As part of the active surveillance, we conducted a bacteriological analysis of faecal specimens from diarrheal patients visiting the Dhaka Hospital of ICDDR, B over a period of 5 months.

MATERIALS AND METHODS

Sample collection and isolation of *E. coli*

Faecal specimens were collected from patients enrolled in the 2% systematic sampling of all patients visiting the Dhaka Hospital of ICDDR, B under its diarrhoeal disease surveillance system, between July and November 2002. Information such as age, sex, and clinical symptoms (type and duration of diarrhoea, dehydration status, and presence of fever and other symptoms) was collected using a standard questionnaire. All the 775 faecal specimens collected from patients in sterile containers were subjected to macroscopic inspection for presence of blood and mucus, and for isolation of enteric bacterial pathogens using standard laboratory methods [21]. From each faecal specimen, three representative lactose-fermenting colonies growing on MacConkey agar (Difco, Detroit, USA) were isolated and identified [21]. The ability of the *stx* harbouring isolates to ferment sorbitol was assessed by streaking of the strains on sorbitol MacConkey Agar (Difco) plates. O-serogroups of *E. coli* isolates were determined by slide agglutination using a commercially available antisera kit (Denka Seiken Co., Tokyo, Japan). All of the *E. coli* isolates were preserved at -80 ℃.

Drug susceptibility tests

Drug susceptibility was determined by the disc diffusion method using the following commercial disks (Hi Media, Mumbai, India), ampicillin (10 μg), chloramphenicol (30 μg), co-trimoxazole (25 μg), ciprofloxacin (5 μg), gentamicin (10 μg), neomycin (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), streptomycin (10 μg), tetracycline (30 μg), cephalothin (30 μg), amikacin (30 μg), furazolidone (50 μg), kanamycin (30 μg) and ceftriaxone (30 μg). The *E. coli* ATCC 25922 strain sensitive to all the drugs was used as a quality control. The characterization of the isolates as susceptible, reduced susceptibility, or resistant was done as recommended by the National Committee for Clinical Laboratory Standards [22].

Screening for virulence genes

PCR for the detection of both chromosomal and plasmid virulence genes was performed using a thermal cycler (Applied Biosystems, Foster City, Calif, USA) in a total volume of 25 μl. The reaction mixture contained 5 μl of culture lysate, 2.5 mM of each dNTP, 30 μM of each primer, 2.5 μl of 10 X PCR Buffer, and 1 U of r-Taq DNA polymerase (Takara, Shuzo, Japan). The primer sequences of the targeted virulence genes and the PCR conditions are presented in Table 1. VTEC-3 (serotype O157:H7 harbouring *stx1* and *stx2* genes) and *E. coli* K-12 strain were used as positive and negative controls, respectively.

Southern hybridisation

The PCR products from 5 representative strains were separated in 1.5% agarose gel and transferred to a Nylon membrane by the capillary method, and the were UV cross-linked (GS Gene linker, Bio-Rad, Hercules, USA). The cloned *stx1* 905-bp DNA fragment from the recombinant plasmid pKTN501 was used as a probe after digestion with *Bam*HI and *Eco*RI. Hybridisation was done using the DIG-DNA Labelling and Detection Kit (Boehringer-Mannheim, Germany).

Sequencing of stx-PCR amplicon

The *stx1* gene was amplified using primers (EVT1 and EVT2). The PCR products were purified by QIA Quick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced directly in an ABI310 automated sequencer (Applied Biosystems) using Big-dye terminator chemistry, and

Table 1. Polymerase Chain Reaction (PCR) primers and conditions used in the study

Primer	Nucleotide sequence of primers (5'-3')	Target	PCR conditions[a]			Amplicon (bp)	Reference
			Denaturing	Annealing	Extension		
EVT1/ EVT2	CAACACTGGATGATCTCAG CCCCCTCAACTGCTAATA	*stx1*	94 ℃, 60s	55 ℃, 60s[b]	72 ℃, 60s	349	48
EVS1/ EVC2	ATCAGTCGTCACTCACTGGT CTGCTGTCACAGTGACAAA	*stx2*	94 ℃, 30s	55 ℃, 60s[b]	72 ℃, 60s	110	48
hlyA1/ hlyA4	GGTGCAGCAGAAAAAGTTGTAG TCTCGCCTGATAGTGTTTGGTA	EHEC *hlyA*	94 ℃, 30s	57 ℃, 60s	72 ℃, 90s	1551	49
wkatB /wkatF	CTTCCTGTTCTGATTCTTCTGC AACTTATTTCTCGCATCATCC	*katP*	94 ℃, 30s	56 ℃, 60s	72 ℃, 90s	2125	50
D1/ D13R	CGTCAGGAGGATGTTCAG CGACTGCACCTGTTCCTGATTA	*etpD*	94 ℃, 30s	52 ℃, 60s	72 ℃, 70s	1062	51
EAE1/ EAE2	AAACAGGTGAAACTGTTGCC CTCTGCAGATTAACCTCTGC	*eae*	94 ℃, 60s	55 ℃, 90s	72 ℃, 90s	350	52
SAAF SAAR	CGTGATGAACAGGCTATTGC ATGGACATGCCTGTGGCAAC	*saa*	94 ℃, 30s	52 ℃, 60s	72 ℃, 40s	119	14

[a]Unless stated, PCR was done for 30 cycles.
[b]After 35 cycles, a final extension step of 10 min at 72 ℃ was performed.

Table 2. Clinical manifestation of patients infected with STEC.

Age Group (Y)	No. (%) STEC infected cases (n=20)	Presenting feature (%) (n=20)		
		Watery Stool	Vomiting	Dehydration
< 5yrs	11 (55)	11 (100)	8 (72)	4 (36)
> 5yrs	9 (45)	6 (67)	5 (55)	6 (67)

the obtained sequences were compared with published *stx1* gene sequences in the GenBank databases using the Blast search program of NCBI.

Vero cell assay

All of the STEC strains were cultured in L-broth (Difco) at 37 ℃ overnight with constant shaking and pelleted by centrifugation at 5000 rpm for 5 min at 4 ℃. Cell pellets were washed and sonicated with Handy Sonic (TOMY, Tokyo, Japan). After centrifugation, the supernatant and the cell lysate were filter-sterilized using 0.22 µm filters (Millex, Millipore, Bedford, USA) and used in the assay. The cytotoxic effect of the STEC strains was assayed on Vero-cells in 96-well flat bottom tissue culture plates (NUNC, Intermad, Denmark) as described elsewhere [23]. The cells were observed microscopically up to 72 hrs for cytotoxicity. VTEC-3 and DH5α were used as positive and negative controls, respectively.

RAPD Analysis

Molecular typing of *stx*-harbouring isolates was done by RAPD-PCR using a single primer 1247 (5'-AAGAGCC CGT-3') [19] in the Gene Amp PCR system 9700 (Applied Biosystems). The PCR mixture was made to a volume of 50 µl containing 100 ng of genomic DNA, 200 µM each dNTP, 30 pmol of primer, 5 µl of 10X PCR buffer, 3 mM $MgCl_2$ and 2.0 U of Taq DNA polymerase (Takara). After a hot start at 80 ℃ for 5 minutes, the DNA was subjected to 35 cycles of denaturing at 94 ℃ for 1 minute, annealing at 40 ℃ for 1 minute, and extension at 72 ℃ for 2 minutes. A final extension step was done for 10 minutes at 72 ℃. The ethidium bromide stained gels were digitised for comparison and to ascertain the clonal relationship between isolates.

Statistical analysis

To determine the statistical significance of diarrhoea among STEC infected patients in different age groups, we tested the data with Chi-square using 2x2 table in EpiInfo 2000, and Fischer Exact test was done to obtain the significance (p) value.

RESULTS

Clinical manifestation

No apparent predilection in the incidence of STEC was observed between patients of different age groups from whom *stx*-harbouring *E. coli* were isolated. When patients excreting *stx*-harbouring *E. coli* were stratified by age, we observed that watery diarrhoea was more common in the <5 year age group as compared to the >5 year of age group (p= 0.07). Though not significant, a similar trend was observed for vomiting and degree of dehydration (Table 2). Patients found to be carrying *stx*-harbouring *E. coli* were not co-infected with other enteric pathogens.

Table 3. Genotypic and phenotypic characteristics of the STEC strains isolated from hospitalized diarrhoea patients.

Strain	Serotype	Virulence gene								Phenotypic characteristics
		Chromosomal					Plasmid			
		stx$_1$	*stx*$_2$	*eae*	*saa*	*hlyA*	*katP*	*etpD*	SF[a]	Antibiogram
J1	O36a	+	-	-	-	-	-	-	-	AChFxKTCCiCoNaSN
J2	O127a	+	-	+	-	-	-	-	-	AChFxCoNa
J3	O6	+	+	-	-	-	-	-	-	AChFxTCCoNaS
J4	O63	+	-	-	-	-	-	-	-	AChCfTCoNaS
J5	O146	+	-	-	-	-	-	-	+	AChGCfFxKTCiCoNa
J6	O78	+	-	-	-	-	-	-	+	AChGFxTCoNaS
J7	ONT	+	-	+	-	-	-	-	+	ACh
J8	O44	+	-	-	-	-	-	-	+	AChCfTCCoNa
J9	O44	+	-	-	-	-	-	-	+	AChTCoNaS
J10	O8	+	-	-	-	-	-	-	+	AChCfTCoNaS
J11	Rough	+	-	-	-	-	-	-	+	AChCfTCCoNa
J12	O27	+	-	-	-	-	-	-	+	AChCfCCoNa
J13	O20	+	-	-	-	-	-	-	+	AChCfTCCiNa
J14	ONT	+	-	-	-	-	-	-	+	AChCfTCCiCoNa
J15	O25	+	-	-	-	-	-	-	+	AChCfKCoNa
J16	ONT	+	-	-	-	-	-	-	+	
J17	O44	+	-	-	-	-	-	-	+	AChTN
J18	O167	+	-	-	-	-	-	-	+	FxTCoS
J19	O15	+	-	-	-	-	-	-	+	AChTCoNa
J20	ONT	+	-	-	-	-	-	-	+	AChTCoS

[a]sorbitol fermentative

Abbreviations: A, ampicillin; Ch, cephalothin; G, gentamicin, Cf, ciprofloxacin, Fx, furazolidone; K, kanamycin; T, tetracycline; C, chloramphenicol; Ci, ceftriaxone; Co, co-trimoxazole; Na, nalidixic acid; S, streptomycin; N, neomycin.

Prevalence of STEC and characterization of the strains

During the study period, 775 faecal specimens were examined from diarrhoeal patients of various age groups. *E. coli* was cultured from 189 (24.4%) of the samples. Twenty *E. coli* isolates were positive (10.6%) in the *stx*-PCR assay indicating an overall incidence rate of 2.6%. All the *stx* harbouring strains were negative for O157 antiserum but belonged to 13 other O serogroups (Table 3). Among the 15 typable strains, O44 serogroup was common (20%) and 4 were not typable (ONT). All except 4 of the STEC strains were found to ferment sorbitol on MacConkey agar (Table 3).

Drug susceptibility

Most of the tested strains were resistant to ampicillin and cephalothin (90% each), co-trimoxazole (80%), tetracycline and nalidixic acid (75% each), ciprofloxacin (45%), streptomycin (40%), chloramphenicol (35%) and furazolidone (30%). The strain J16 was susceptible to all the tested drugs (Table 3). None of the isolates was resistant to amikacin or norfloxacin. As shown in Table 3, there was no common resistance pattern among the 20 strains tested.

Vero cell assay

In the Vero cell cytotoxic assay, except for VTEC-3 (positive control strain), none of the *stx* harbouring strains exhibited the characteristic cytotoxicity.

Detection of virulence genes and molecular typing

In the multiplex PCR assay, we identified 20 *E. coli* strains harbouring *stx*, 19 carrying the *stx1* and one carrying both *stx1* and *stx2* (Table 3). To confirm the authenticity of the PCR products, Southern hybridization was performed with DIG-labelled cloned probe from pKTN501. In addition, the amplified *stx1* gene of representative strains was sequenced and the sequence showed 100% homology with published *stx1* gene sequences. We identified two strains (J 2, J7) that had the *eae* (Table 3). None of the 20 *stx*-harbouring strains were positive for *hlyA, kat, etpD* or *saa* genes in PCR assays.

The *stx*-harbouring isolates were characterized by RAPD-PCR to analyse the genetic relatedness. All of the strains showed multiple amplicons with fragment sizes ranging from 0.5 to 6.0 kb. The RAPD profile of the STEC strains showed heterogeneous banding patterns (Fig. 1).

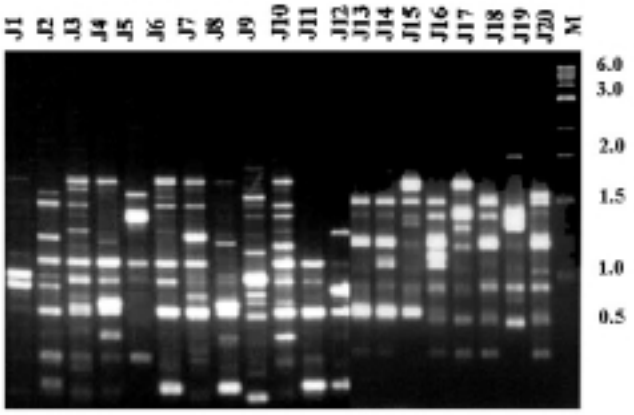

Fig. 1. RAPD-PCR results of stx_1-harbouring *E. coli* isolates. (M, Molecular weight marker (Kb); J1to J20 STEC strains isolated from the clinical diarrhoea patients).

DISCUSSION

To our knowledge, this is the first report on the isolation of *E. coli* strains harbouring *stx* from diarrhoeal patients in Bangladesh. STEC was not associated with diarrhoea in a previous study conducted in this region in 1991-92 [24]. This study revealed a low prevalence of non-O157 *stx1*-harbouring *E. coli* in Bangladesh, and is unlike the reports from Europe, Australia, Brazil and Argentina [25-28] where a much higher incidence of non-O157 STEC was observed. However, the overall incidence of STEC is much lower in developing countries as compared to industrialized countries. The most frequent serotype observed in this study was O44 (20%).

Multidrug resistance was frequent among STEC strains encountered in this study, especially to ampicillin, cephalothin, co-trimoxazole, tetracycline and nalidixic acid. In this study, 45% of the STEC strains were resistant to ciprofloxacin. Due to excessive use of this drug in recent years, many enteric pathogens were reported to be resistant to ciprofloxacin in India [29-32]. Another interesting observation encountered in the present study is the incidence of a higher number of ciprofloxacin-resistant than norfloxacin-resistant STEC strains. The same trend was reported for *V. cholerae* O1 [29] and *Aeromonas* spp. [32]. Even though ciprofloxacin and norfloxacin are basically the same drug, extensive use of the former and a difference in accumulation kinetics between the two are thought to account for the variation in resistance pattern [29].

Analysis of virulence markers indicated that the majority of the non-O157 strains carried *stx1*, whereas the *eae* gene was detected at a very low frequency. Non-O157 STEC strains harbouring *stx1* but lacking *stx2* have been reported earlier [2, 33]. The higher prevalence of *E. coli* with *stx1* genotype was also noted in a study on STEC in the neighbouring region, Kolkata, India [19]. In this study, only two strains harboured the *eae* gene. An increasing number of reports indicate that production of intimin is not essential for the STEC-mediated pathogenesis [18, 34, 35]. One study reported toxin expression by intimin-negative STEC strains (O91:H21 isolates) using only 50% lethal dose in a streptomycin-treated CD-1 mouse model [36]. A subset of human STEC, such as *stx1c*-harbouring strains, generally lacks the intimin-encoding *eae* gene [35, 37]. The factors responsible for colonization by non-O157 strains lacking the intimin are not well established, although many candidate adhesins have recently been reported [14, 38-41]. The STEC strains carrying *eae* are generally considered to have higher virulence for humans than those lacking the *eae* [42]. However, from the available reports [34,43] it is evident that the association between HUS and the absence of *eae* remains unclear.

Recently, Paton and Paton [14] described a novel megaplasmid-encoded adhesin (*saa*) in human STEC lacking the *eae* gene. As most of the *stx1*-harbouring *E. coli* strains were negative for *eae* in this study, we included the *saa* in the investigation for plasmid-associated virulence markers along with other possible additional virulence traits such as STEC haemolysin (*hlyA*), a bifunctional catalse peroxidase (*KatP*), and the *etpD* gene cluster, which encodes a type II secretion pathway [9, 10]. None of the tested isolates produced the specific amplicons.

To confirm the authenticity of *stx* PCR results, we confirmed that the amplicons were specific for *stx1* by Southern hybridisation assay with *stx1*-specific probes prepared from the plasmid pKTN501. The PCR products showed positive signals in the hybridisation assay, confirming that the PCR assay was specific for *stx1*. In a bid to further confirm the presence of *stx1*, we performed sequence analysis of the corresponding *stx1* PCR product and observed 100% homology to the published *stx1* sequences in the database.

A paradoxical observation in this study was that none of the *stx* harbouring strains demonstrated the production of Stx in the Vero cell cytotoxicity assay. A similar observation was made in a study on *stx1* strains with low toxin expression isolated in Thailand [18]. It is a well established fact that the genes encoding the Stx is located in the genomes of temperate bacteriophages [44] and that the phage encodes transcription factor essential to activation of Shiga toxin expression [45]. Recent findings suggest that the *stx* harbouring strains have weak or ineffective phage transcription factor leading to sub-optimal or no production of Stx [46]. It appears that the role of Stx in causing watery diarrhoea is non-essential as evidenced in an earlier study [47].

Our study has, for the first time in Bangladesh, demon-

strated the existence of *stx*-harbouring *E. coli*. The role of *stx1*-harbouring *E. coli* isolates, which lack *eae*, *saa* and other virulence markers, needs to be studied in further detail. The lack of established virulence genes and the non-expression of Stx in our set of *E. coli* strains make it difficult to establish their role in diarrhoea. Our observation of clinical variations in different age groups could not be generalized due to the small sample size. However, the fact that non-O157 STEC are present in the Bangladeshi setting underscore the need to conduct a well-designed study to determine the significance of STEC as a pathogen in this part of the world.

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REFERENCES

1. Karmali MA. Infection by verocytotoxin-producing *Escherichia coli.* Clin Microbiol Rev 1989; 2: 15-38.
2. Boerlin P, McEwen SA, Boerlin-Petzold F, Wilson JB, Johnson RP, Gyles CL. Association between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. J Clin Microbiol 1999; 37: 497-503.
3. Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, *et al.* Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med 1983; 308: 681-85.
4. O'Brien AD, Holmes RK. Shiga and Shiga-like toxin. Microbiol Rev 1987; 1: 206-20.
5. O'Brien AD, Tesh VL, Rolfe AD,Jackson MP, Olsnes S, Sandvig K, et al. Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis. Curr Top Microbiol Immunol 1992; 180: 65-94.
6. Strockbine NA, Marques LR, Newland JW, Smith HW, Holmes RK, O'Brien AD. Two toxin-converting phages from *Escherichia coli* O157:H7 strain 933 encode antigenically distinct toxins with similar biologic activities. Infect Immun 1986; 53: 135-40.
7. Jackson MP, Newland JW, Holmes RK, O'Brien AD. Nucleotide sequence analysis of the structural genes for Shiga-like toxin I encoded by bacteriophage 933J from *Escherichia coli.* Microb Pathog 1987; 2: 147-53.
8. Kaper JB, Elliott S, Sperandio V, Perna NT, Mayhew GF, Blattner FR. Attaching and effacing intestinal histopathology and the locus of enterocyte effacement. In: Kaper JB, O'Brien AD, eds. *Escherichia coli* and other Shiga toxin-producing *E. coli* strains. Washington. DC: American Society for Microbiology, 1998; 163-82.
9. Schmidt H, Beutin L, Karch H. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. Infect Immun 1995; 63: 1055-61.
10. Brunder W, Schmidt H, Karch H. KatP. A novel catalase-peroxidase encoded by the large plasmid of enterohaemorrhagic *Escherichia coli* O157:H7. Microbiology1996; 142: 3305-15.
11. Schmidt H, Henkel B, Karch H. A gene cluster closely related to type II secretion pathway operons of Gram-negative bacteria is isolated on the large plasmid of enterohemorrhagic *Escherichia coli* O157 strains. FEMS Micro Lett 1997; 148: 265-72.
12. Brunder W, Schmidt H, Karch H. EspP, a novel extracellular serine protease of enterohaemorrhagic *Escherichia coli* O157:H7 cleaves human coagulation factor V. Mol Microbiol 1997; 24: 767-78.
13. Schmidt H, Geitz C, Phillips IT, Matthias F, Karch H. Non-O157 pathogenic Shiga toxin-producing *Escherichia coli*: phenotypic and genetic profiling of virulence traits and evidence for clonality. J Infect Dis 1999; 179: 115-23.
14. Paton AW, Paton JC. Direct detection and characterization of Shiga toxigenic *Escherichia coli* by multiplex PCR for *stx1, stx2, eae, ehxA,* and *saa.* J Clin Microbiol 2002; 40: 271-74.
15. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli.* Clin Microbiol Rev 1998; 11: 142-201. Erratum in: Clin Microbiol Rev 11: 403.
16. Jelacic JK, Damrow T, Chan GS, Jelacic S, Bielaszenwska M, Ciol M et al. Shiga toxin-producing *Escherichia coli* in Montana : Bacterial genotypes and clinical profiles. J Infect Dis 2003; 188: 719-29.
17. Chakraborty S, Khan A, Kahali S, Faruque SM, Yamasaki S, Ramamurthy T. Infantile diarrhoea associated with sorbitol-fermenting, non-Shiga toxin-producing *Escherichia coli* O157:H$^-$. Eur J Clin Microbiol Infect Dis 2003; 22: 324-26.
18. Leelaporn A, Phengmak M, Eampoklap B, Manatsathit S, Tritilanunt S, Siritantikorn S, et al. Shiga toxin- and enterotoxin-producing *Escherichia coli* isolated from subjects with bloody and non-bloody diarrhea in Bangkok, Thailand. Diagn Microbiol Infect Dis 2003; 46: 173-180.
19. Khan A, Das SC, Ramamurthy T, Sikdar A, Khanam J, Yamasaki S, et al. Antibiotic resistance, virulence gene, and molecular profiles of Shiga toxin-producing *Escherichia*

coli isolates from diverse sources in Culcutta, India. J Clin Microbiol 2002; 40: 2009-15.
20. Strockbine NA, Faruque SM, Kay BA, Haider K, Alam K, Alam AN, Tzipori S, Wachsmuth IK. DNA probe analysis of diarrhoeagenic *Escherichia coli*: detection of EAF-positive isolates of traditional enteropathogenic *E. coli* serotypes among Bangladeshi paediatric diarrhoea patients. Mol Cell probes 1992; 6(2): 93-99.
21. World Health Organization. Manual for laboratory investigation of acute enteric infections 1987; CDD/83.3: WHO, Geneva, Switzerland.
22. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing, 12th informational supplement. National Committee for Clinical Laboratory Standards document 2002; M100-12. NCCLS, Wayne, Pa.
23. Kurazono H, Sasakawa C, Yoshikawa M, Takeda Y. Cloning of a Vero toxin (VT1, Shiga-like toxin1) gene from a VT1-converting phage isolated from *Escherichia coli* O 157:H7. FEMS Microbiol Lett 1987; 44:23-26.
24. Albert MJ, Faruque SM, Faruque ASG, Neogi PKB, Ansaruzzaman M, Bhuiyan NA, et al. Controlled study of *Escherichia coli* diarrheal infections in Bangladeshi children. J Clin Micro 1995; 33: 973-77.
25. Caprioli A, Tozzi AE. Epidemiology of Shiga toxin-producing *Escherichia coli* infections in continental Europe. *In*: Kaper, J.B., and A.D. O'Brien, editors. *Escherichia coli* and other Shiga toxin-producing *E. coli* strains. Washington, DC: American Society for Microbiology; 1998. p.38-48.
26. Elliott EJ, Robins-Browne RM, O'Loughlin EV, Bennett-Wood V, Bourke J, Henning P, et al. Contributors to the Australian Paediatric Surveillance Unit. Nationwide study of haemolytic uraemic syndrome: clinical, microbiological, and epidemiological features. Arch Dis Child 2001; 85: 125-31.
27. Vaz TM, Irino K, Kato MA, Dias AM, Gomes TA, Medeiros MI, et al. Virulence properties and characteristics of Shiga toxin-producing *Escherichia coli* in Sao Paulo, Brazil, from 1976 through 1999. J Clin Microbiol 2004; 42: 903-05.
28. Parma AE, Sanz ME, Blanco JE, Blanco J, Vinas MR, Blanco M, et al. Virulence genotypes and serotypes of verotoxigenic *Escherichia coli* isolated from cattle and foods in Argentina. Importance in public health. Eur J Epidemiol 2000; 16: 757-62.
29. Garg P, Sinha S, Chakraborty R, Bhattacharya SK, Nair GB, Ramamurthy T et al.. Emergence of fluoroquinolone-resistant strains of *Vibrio cholerae* O1 biotype El Tor among hospitalized patients with cholera in Calcutta, India, J Antimicrob. Agents Chemother 2001; 45: 1605-06.
30. Chakraborty S, Deokule JS, Garg P, Bhattacharya SK, Nandy RK, Nair GB, et al. Concomitant infection of enterotoxigeneic *Escherichia coli* in an outbreak of cholera caused by *Vibrio cholerae* O1, O139 in Ahmedabad, India. J Clin Microbiol 2001; 39: 3241-46.
31. Pazhani GP, Sarkar B, Ramamurthy T, Bhattacharya SK, Takeda Y, Niyogi SK. Clonal multidrug-resistant *Shigella dysenteriae* type 1 strains associated with epidemic and sporadic dysenteries in eastern India. Antimicrob Agents Chemther 2004; 48: 681-84.
32. Sinha S, Chattopadhyay S, Bhattacharya SK, Nair GB, Ramamurthy T. An unusually high level of quinolone resistance-determining regions of *Aeromonas caviae* isolated from diarrhoeal patients. Res Microbiol 2004; 155: 827-29.
33. Welinder-Olsson C, Badenfors M, Cheasty T, Kjellin E, Kaijser B. Genetic profiling of enterohemorrhagic *Escherichia coli* strains in relation to clonality and clinical signs of infection. Clin Microbiol 2002; 40: 959-64.
34. Paton AW, Woodrow MC, Doyle RM, Lanser JA, Paton JC. Molecular characterization of a Shiga toxigenic *Escherichia coli* O113:H21 strain lacking *eae* responsible for a cluster of cases of hemolytic-uremic syndrome. J Clin Microbiol 1999; 37: 3357-61.
35. Zhang W, Bielaszewska M, Kuczius T, Karch H. Identification, characterization, and distribution of a Shiga Toxin 1 gene variant (stx_{1c}) in *Escherichia coli* strains isolated from humans. J Clin Microbiol 2002; 40: 1441-46.
36. Melton-Celsa AR, Darnell SC, O'Brien AD. Activation of Shiga-like toxins by mouse and human intestinal mucus correlates with virulence of enterohemorrhagic *Escherichia coli* O91:H21 isolates in orally infected, streptomycin-treated mice. Infect Immun 1996; 64: 1569-76.
37. Friedrich AW, Borell J, Bielaszewska M, Fruth A, Tschape H, Karch H. Shiga toxin 1c-producing *Escherichia coli* strains: phenotypic and genetic characterization and association with human disease. J Clin Microbiol 2003; 4: 2448 -53.
38. Doughty S, Sloan J, Bennett-Wood V, Robertson M, Robins-Browne RM, Hartland EL. Identification of a novel fimbrial gene cluster related to long polar fimbriae in locus of enterocyte effacement-negative strains of enterohemorrhagic *Escherichia coli.* Infect Immun 2002; 70: 6761-69.
39. Toma C, Matinez Espinosa E, Song T, Miliwebsky E, Chinen I, Iyoda S et al. . Distribution of putative adhesins in different seropathotypes of Shiga toxin-producing *Escherichia coli.* J Clin Microbiol 2004; 42: 4937-46.
40. Stevens MP, Roe AJ, Vlisidou I, van Diemen PM, La Ragione RM, Best A et al. Mutation of *toxB* and a truncated version of the *efa-1* gene in *Escherichia coli* O157:H 7 influences the expression and secretion of locus of enterocyte effacement-encoded proteins but not intestinal colonization in calves or sheep. Infect Immun 2004; 72: 5402-11.
41. Brunder W, Khan AS, Hacker J, Karch H. Novel type of fimbriae encoded by the large plasmid of sorbitol-fermenting enterohemorrhagic *Escherichia coli.* Infet Immun 2001; 69: 4447-57.
42. Barret TJ, Kaper JB, Jerse AE, Wachsmuth IK. Virulence factors in Shiga-like toxin-producing *Escherichia coli* isolated from humans and cattle. J Infect Dis 1992; 165: 979-

80.
43. Centres for Disease Control and Prevention. Outbreak of acute gastroenteritis attributable to *Escherichia coli* serotype O104:H21-Helena, Montana, 1994. Morbid Mortal Weekly 1995; 44: 501-03.
44. O'Brien AD, Newland JW, Miller SF, Holmes RK, Smith HW, Formal SB. Shiga-like toxin converting phages from *Escherichia coli* strains that cause hemorrhagic colitis or infantile diarrhea. Science 1984; 226: 694-96.
45. Neely MN, Friedman DI. Arrangement and functional identification of genes in the regulatory region of lambdoid phage H-19B, a carrier of a Shiga-like toxin. Gene 1998; 223: 105-13.
46. Wagner PL, Acheson DW, Waldor MK. Isogenic lysogens of diverse Shiga toxin 2-encoding bacteriophages produce markedly different amounts of Shiga toxin. Infect Immun 1999; 67: 6710-14.
47. Tzipori S, Karch H, Wachsmuth KI, Robins-Browne RM, O'Brien AD, Lior H, et al. Role of a 60-megadalton plasmid and Shiga-like toxins in the pathogenesis of infection caused by enterohemorrhagic *Escherichia coli* O157:H7 in gnotobiotic piglets. Infect Immun 1987; 55: 3117-25.
48. Pal A, Ghosh S, Ramamurthy T, Yamasaki S, Tsukamoto T, Bhattacharya SK, et al. Shiga toxin-producing *Escherichia coli* from healthy cattle in a semi-urban community in Clacutta, India. Indian J Med Res 1999; 110: 83-85.
49. Schmidt H, Beutin L, Karch H. Molecular basis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. Infect. Immun. 1995; 63: 1055-61.
50. Brunder W, Schmidt H, Karch H. KatP, a novel catalase-peroxidase encoded by the large plasmid of enterohaemorrhagic *Escherichia coli* O157:H7. Microbiology 1996; 142: 3305-15.
51. Schmidt H, Henkel B, Karch H. A gene cluster closely related to type II secretion pathway operons of gram-negative bacteria is located on the large plasmid of enterohemorrhagic *Escherichia coli* O157 strains. FEMS Microbiol Lett 1997; 148: 265-72.
52. Yu J, Kaper JB. Cloning and characterization of the *eae* gene of enterohemorrhagic *Escherichia coli* O157:H7. Mol. Microbiol. 1992; 6: 411-77.