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 sau 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 sequelae 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-
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 cytotoxin 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.microbiomed.com.au/frames/feature/ets/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/whocssrahp988.c.html). 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 diarrhea, 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°C.

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 a 1.5% agarose gel and transferred to a Nylon membrane by the capillary method, and the were UV crosslinked (GS Gene linker, Bio-Rad, Hercules, USA). The cloned stxl 905-bp DNA fragment from the recombinant plasmid pKTN501 was used as a probe after digestion with BamHI and EcoRI. Hybridisation was done using the DIG-DNA Labelling and Detection Kit (Boehringer-Mannheim, Germany).

Sequencing of stx-PCR amplicon

The stxl 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
the obtained sequences were compared with published stxl 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°C overnight with constant shaking and pelleted by centrifugation at 5000 rpm for 5 min at 4°C. 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 MgCl₂ and 2.0 U of Taq DNA polymerase (Takara). After a hot start at 80°C for 5 minutes, the DNA was subjected to 35 cycles of denaturing at 94°C for 1 minute, annealing at 40°C for 1 minute, and extension at 72°C for 2 minutes. A final extension step was done for 10 minutes at 72°C. 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.
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 tested drugs (Table 3). None of the isolates was resistant to streptomycin (40%) and 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 *stxl* and one carrying both *stxl* 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 (J2, J17) that had the *eae* (Table 3). None of the 20 *stx*-harbouring strains was 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).

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Chromosomal virulence gene</th>
<th>Plasmid virulence gene</th>
<th>Phenotypic characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O36a</td>
<td><em>stx</em>, <em>eae</em></td>
<td><em>kat</em>, <em>etpD</em></td>
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</tr>
<tr>
<td>2</td>
<td>O127a</td>
<td><em>stx</em>, <em>eae</em></td>
<td><em>kat</em>, <em>etpD</em></td>
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<tr>
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<td>O6</td>
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<td><em>kat</em>, <em>etpD</em></td>
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</tr>
<tr>
<td>4</td>
<td>O63</td>
<td><em>stx</em>, <em>eae</em></td>
<td><em>kat</em>, <em>etpD</em></td>
<td><strong>+</strong></td>
</tr>
<tr>
<td>5</td>
<td>O146</td>
<td><em>stx</em>, <em>eae</em></td>
<td><em>kat</em>, <em>etpD</em></td>
<td><strong>+</strong></td>
</tr>
<tr>
<td>6</td>
<td>O78</td>
<td><em>stx</em>, <em>eae</em></td>
<td><em>kat</em>, <em>etpD</em></td>
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<tr>
<td>7</td>
<td>ONT</td>
<td><em>stx</em>, <em>eae</em></td>
<td><em>kat</em>, <em>etpD</em></td>
<td><strong>+</strong></td>
</tr>
<tr>
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<td>O44</td>
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<td><strong>+</strong></td>
</tr>
<tr>
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<td>O44</td>
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<td><em>kat</em>, <em>etpD</em></td>
<td><strong>+</strong></td>
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<td><em>kat</em>, <em>etpD</em></td>
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</tr>
<tr>
<td>11</td>
<td>Rough</td>
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<td><em>kat</em>, <em>etpD</em></td>
<td><strong>+</strong></td>
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<td><em>kat</em>, <em>etpD</em></td>
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<td><strong>+</strong></td>
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<tr>
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<td><em>kat</em>, <em>etpD</em></td>
<td><strong>+</strong></td>
</tr>
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<td><em>kat</em>, <em>etpD</em></td>
<td><strong>+</strong></td>
</tr>
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<td><em>kat</em>, <em>etpD</em></td>
<td><strong>+</strong></td>
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<tr>
<td>19</td>
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<td><em>stx</em>, <em>eae</em></td>
<td><em>kat</em>, <em>etpD</em></td>
<td><strong>+</strong></td>
</tr>
<tr>
<td>20</td>
<td>ONT</td>
<td><em>stx</em>, <em>eae</em></td>
<td><em>kat</em>, <em>etpD</em></td>
<td><strong>+</strong></td>
</tr>
</tbody>
</table>

*Stx* refers to Shiga toxin genes. **Abbreviations:** A, ampicillin; Ch, cephalothin; G, gentamicin, Cf, ciprofloxacin, Fx, furazolidone; K, kanamycin; T, tetracycline; C, chloramphenicol; Cf, ceftriaxone; Co, co-trimoxazole; Na, nalidixic acid; S, streptomycin; N, neomyacin.

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.
STEC strains harbouring stx1 gene was detected at a very low frequency. Non-O157 eae-negative strains carrying stx1, which is responsible for colonization by non-O157 STEC strains, generally lacks the intimin-encoding eae gene [35, 37]. The factors responsible for colonization by non-O157 STEC strains are not well established, and 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 gene [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 stxl-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 catalase 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 stxl PCR results, we confirmed that the amplicons were specific for stxl. In a bid to further confirm the presence of stxl, we performed sequence analysis of the corresponding stxl PCR product and observed 100% homology to the published stxl sequences in the database.

A paradoxical observation in this study was that none of the stxl-harbouring strains demonstrated the production of Stx in the Vero cell cytotoxicity assay. A similar observation was made in a study on stxl 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 stxl 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-

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 stxl-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, cefalothin, 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 stxl, whereas the eae gene was detected at a very low frequency. Non-O157 STEC strains harbouring stxl but lacking stx2 have been reported earlier [2, 33]. The higher prevalence of E. coli with stxl 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 stxl-harbouring strains, generally lacks the intimin-encoding eae gene [35, 37]. The factors responsible for colonization by non-O157 STEC strains lacking the intimin are not well established, and 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 gene [42]. However, from the available reports [34, 43] it is evident that the association between HUS and the absence of eae remains unclear.

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Fig. 1. RAPD-PCR results of stx1-harbouring E. coli isolates. (M, Molecular weight marker (Kb); J1to J20 STEC strains isolated from the clinical diarrhoea patients).
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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