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MOLECULAR DETECTION AND ANTIBIOGRAM OF SHIGA TOXIN PRODUCING *ESCHERICHIA COLI* (STEC) ISOLATED FROM DIARRHEIC CHILDREN

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ABSTRACT

This study was designed to determine the shiga toxin producing genes and to investigate antibiotic sensitivity or resistant patterns of the *Escherichia coli* isolated from diarrheic children at Mymensingh Medical College Hospital, Bangladesh. A total of 83 stool samples were collected and screened for the detection of *E. coli* on the basis of cultural, staining and biochemical properties followed by molecular detection by Polymerase Chain Reaction (PCR) using genus specific 16SrRNA primers. Antimicrobial susceptibility pattern of *E. coli* was determined by disc diffusion method against 9 antimicrobial agents. In this study, 27 (32.53%) out of 83 samples, were confirmed as *E. coli*. Overall prevalence of shiga toxin producing *E. coli* (STEC) among the examined children was 1.20% (n=1/83). Further, 27 *E. coli* isolates were analyzed for the presence of *Stx-1* and *Stx-2* genes by duplex-PCR. The STEC isolate was confirmed to be positive for the presence of the *Stx-2* gene only. Highest susceptibility of the *E. coli* isolates was found against Gentamicin (92.59%), followed by Ciprofloxacin (48.14%) and Moxifloxacin (33.33%). More than 77.78% of the isolates were resistant to more than three antibiotics thus defined as multi-drug resistant (MDR). In conclusion, Gentamicin and Ciprofloxacin can be recommended as the effective drugs successful treatment of STEC infections in children.

Key words: STEC, children, PCR, Antibiotic sensitivity, duplex PCR, Bangladesh

INTRODUCTION

Shiga toxin producing *Escherichia coli* (STEC) is known as Verotoxin producing *E. coli*. Infections due to STEC that can result in severe bloody diarrhea (hemorrhagic colitis, HC) which may evolve towards the life-threatening hemolytic-uremic syndrome (HUS). Currently six *E. coli* pathotypes are recognized which can cause diarrhea in humans (Turner *et al*., 2006). Few studies have provided required information on the outbreak of disease producing or pathogenic *E. coli* (Higgins *et al.*, 2005).

It is estimated that the diarrheal diseases account for 4.1% of the total daily global burden of diseases and are cause for the deaths of 1.8 million people every year and 90% of them are children under the age of 5 years of old (Islam *et al*., 2006). In addition, diarrheal illnesses responsible for an estimated 12,600 deaths each day in children under 5 years of old in Asia, Africa, and Latin America, especially in developing countries (Alikhani *et al*., 2006). STEC has become a major public health problem for the last few decades. Other strains may cause outbreaks including many waterborne diseases (Leelaporn *et al*., 2003). Sporadic cases and outbreaks have been already reported from many developed countries. STEC infections also have been reported in Latin America, India and some other developing countries (Kaddu-Mulindw *et al*., 2001; Leelaporn *et al*., 2003).

Though STEC has not been established as a major etiological agent of diarrhea in Bangladesh, it has already isolated from diarrheic children, cattle and calves; suggesting that this enteropathogen may cause a serious public health problem (Nazir *et al*., 2005, 2007; Islam *et al*., 2006; Munshi *et al*., 2012; Talukdar *et al*., 2013). STEC has also been reported from the broiler chicken in Bangladesh (Mamun *et al*., 2016). Several studies also showed isolation of shigatoxigenic *E. coli* from water (Talukdar *et al*., 2013) and from urine of the hospitalized patient in Bangladesh (Islam *et al*., 2015).

As per previous reports, it is revealed that several works have been performed for the isolation, identification and molecular characterization of STEC in Bangladesh (Islam *et al*., 2006; Ansari *et al*., 2014; Mamun *et al*., 2016; Jahan *et al*., 2016). However, most of the study patterns were based on surveillance system (Islam *et al*., 2006). Some studies showed isolation of *E. coli* on the basis of biochemical characterization only (Zinnah *et al*., 2007). Ahmed *et al*. (2012) studied with STEC considering both adult and children but not with hospitalized children. The present study was designed to isolate and identify *E. coli* from hospitalized diarrheic children, to detect the presence of virulent gene in the isolated *E. coli*, to determine the prevalence of *E. coli* in hospitalized diarrheic children and to determine the antibiotic sensitivity and resistance pattern of the isolated *E. coli.*

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MATERIALS AND METHODS

Sample collection

The research work was conducted during the period from January 2016 to May 2016 at the Department of Microbiology and Hygiene, Faculty of Veterinary Science (FVS), Bangladesh Agricultural University (BAU), Mymensingh-2202, Bangladesh. Samples of diarrheic stool of the affected hospitalized children were usually available at the Mymensingh Medical College Hospital (MMCH), Mymensingh. A cross-sectional study was designed to investigate the prevalence of the *E. coli* including STEC in diarrhea affected children at MMCH. A total number of 83 stool samples were collected by sterile cotton bud and were put into eppendorf tube containing nutrient broth and brought to the laboratory at the Department of Microbiology and hygiene, BAU by maintaining cool chain. Ages of the children were also recorded.

Study design

The whole experiment was divided into three steps. The steps included isolation of the bacteria from fecal samples of the hospitalized diarrheic children from MMCH. Identification of the *E. coli* by cultural, morphological, biochemical characteristics and PCR. Molecular characterization of STEC by duplex PCR followed by antibiotic sensitivity at the final step.

Cultural identification

Primary growth was performed in nutrient broth followed by inoculation into selective media and incubated at 37^0C for overnight. After primary culture of the organism, a 10-fold dilution was made to reduce overgrowth of the organisms. After that 100 µl was inoculated onto Mac-Conkey agar. The colonies showing typical cultural characteristics of *E. coli* were selected for subculture on selective media such as Eosin Methylene Blue (EMB). The colonies showing typical characteristics of *E. coli* onto EMB agar to confirm the isolates as *E. coli.* Gram's staining and a series of biochemical tests were also performed.

Method of extraction of genomic DNA by boiling method

The genomic DNA of each *E. coli* isolate was extracted by boiling method. Single colony of each isolate was inoculated into 200 µl of distilled water followed by boiling for 10 min. After boiling the samples were immediately kept on ice for few minutes for cold shock. Finally centrifugation was done at 10000 rpm for 10 min. The supernatant was collected and used as DNA template for PCR.

Amplification of 16SrRNA and *Stx-1* and *Stx-2* genes in *E. coli* by PCR

To amplify 16SrRNA of *E. coli* genus specific primers were used (Table 1). The total volume of PCR mixture was 25 µl consisting of 12.5 µl PCR master mixture, 1 µl of each primers, 5 µl of template DNA. The thermal profile of PCR for 16SrRNA was 95^0C, 5 min for initial denaturation, 94^0C, 30 sec for denaturation, 58^0C, 1 min for annealing, 72^0C, 1 min for elongation and 72^0C, 10 min for final extension and the holding temperature was 4^0C. The thermal profile of PCR for *Stx*-1 and *Stx*-2 were 95^0C, 5 min for initial denaturation, 94^0C, 30 sec for denaturation, 56^0C, 1 min for annealing, 72^0C, 1 min for elongation and 72^0C, 10 min for final extension and the holding temperature was 4^0C.

Table 1. Primers used in this study with sequences

Primer Name	Gene Targeted	Primer Sequence (5´-3´)	Amplicon size (bp)	Reference
EC16SrRNA F	16SrRNA	5´GACCTCGGTTTAGTTCACAGA3´	585	Schippa *et al.* (2010)
EC16SrRNA R		5´CACACGCTGACGCTGACCA3´		
EC *Stx*-1 F	*Stx*-1	5´CACAATCAGGCGTCGCCAGCGCACTTGCT3´	606	Heuvelink *et al*. (1995)
EC *Stx*-1R		5´TGTTGCAGGGATCAGTCGTACGGGGATGC3´		
EC *Stx*-2 F	*Stx*-2	5´CCACATCGGTGTCTGTTATTAACCACACC3´	372	Heuvelink *et al*. (1995)
EC *Stx*-2R		5´GCAGAACTGCTCTGGATGCATCTCTGGTC3´		

F=Forward; R=Reverse; bp= Base pair

Antibiotic sensitivity test by the disc diffusion method

The disc diffusion method was used to detect antimicrobial susceptibility assay according to the recommendation of Clinical and Laboratory Standards Institute (CLSI) (formerly National Committee for Clinical Laboratory Standards (CLSI, 2013). Susceptibility of *E. coli* isolates to 9 mostly prescribed antimicrobial agents (Table 3) were measured *in vitro* by employing the modified Kirby-Bauer method (Bauer *et al*., 1966).

RESULTS AND DISCUSSION

Initially, the *E. coli* were screened on the basis of characteristics colony morphologies on Mac-Conkey agar. Out of 83, 27 samples were suspected as *E. coli* based on fermentation of lactose on Mac-Conkey agar and development of bright pink or red colonies (Table 2). Each sample was then sub-cultured onto EMB agar. All 27 suspected *E. coli* isolates produced greenish-black colonies with metallic sheen on EMB agar. The pure cultures of suspected *E. coli* isolates were subjected for Gram staining. In Gram's staining method, the organisms were found as Gram-negative, small rod shaped, arranged in single or paired. In sugar fermentation tests, all the isolates produced both acid and gas by fermentation of sugars indicated by color change and deposition of gas in Durham's tube.

Table 2. Cultural characteristics and overall prevalence of 16SrRNA , *Stx*-2 gene

Source of samples	No. of total samples	No. of positive samples	No. of 16SrRNA Positive samples	*Stx*-1 Positive	*Stx*-2 Positive	No. (%) of 16SrRNA	No. (%) of *Stx-1*	No. (%) of STEC among sampled patients	No. (%) of *Stx*-2 among isolates
MMCH	83	27	27	0	1	27 (32.53%)	0	1 (1.20%)	1 (3.70%)

All the isolates were methyl-red (MR) positive, VP negative, indole positive, which are indicative of the identification of *E. coli*. All the 27 isolates of *E. coli* showed production of oxygen bubbles indicative for positive result in catalase test. All the isolates of *E. coli* which were presumptively identified on the basis of cultural, Gram's staining and biochemical tests were confirmed by Polymerase chain reaction using genus specific 16SrRNA primers (Figure 1). A total of 27 isolates were confirmed as *E. coli* by amplifying genus specific 16SrRNA primers. Out of 27 isolates of *E. coli* only one sample was found positive for *Stx-2* gene (Table 2, Figure 2). But no samples were found *Stx-1* positive. In duplex-PCR, 372 bp sized amplicon of *Stx-2* genes were amplified successfully.

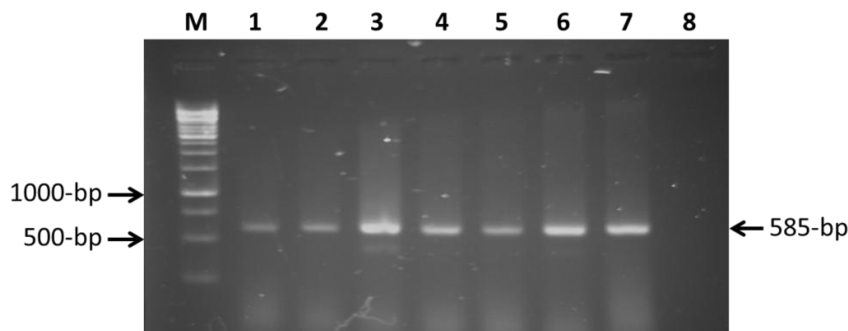

Figure 1. Amplification of 16SrRNA (585 bp) specific genomic primer; Lane 1: 1 kbp DNA ladder, Lane 2-7: positive for 16srRNA; Lane 8: Positive control; Lane 9: Negative control

Out of 83, 27 isolates were found to be positive for 16SrRNA genes. So, overall prevalence of *E. coli* was 32.53%. Among the positive isolates, only 1 isolate (3.70%) was *Stx*-2 positive. Overall prevalence of the STEC from the diarrheic children was detected 1.20%.

All 27 *E. coli* isolates were tested against 9 antibiotics which are frequently suggested by the pediatricians of the MMCH (Table 3). Among these, Gentamicin showed highest susceptibility (92.59%), which followed Ciprofloxacin (48.14%) and Moxifloxacin (33.33%), respectively (Table 3). Also, Ceftriaxone (29.63%) and Cefixime (29.63%) both were found as moderately sensitive. Highest resistant pattern was showed by Amoxycillin (88.88%), Azithromycin (85.18%), and Cephradine (85.18%), followed by Ceftriaxone and Levofloxacin (70.37%), Cefixime (62.96%), Ciprofloxacin (29.62%) and Gentamicin (7.40%), respectively (Table 3). More than 77.78% of the isolates were resistant to at least three or more antibiotics, thus defined as multi-drug resistant (MDR) (Table 4).

This study revealed the prevalence of *E. coli* was 32.53%, as supported by Talukdar *et al*. (2013) who reported the rate as 36%. In our study, on the basis of virulence, the prevalence was 3.70% of *Stx*-2 only. Prevalence of *Stx*-2 among the total samples was 1.20%. This result was inclined with the findings of Islam *et al*. (2006) who reported that 2.2% children were infected with STEC. On the other hand, Dhanashree and Mallya (2008) could detect only 1 *Stx-2* gene among 140 stool samples. A study in Bangladesh conducted by Albert *et al*. (1995) showed that STEC was not present in any diarrheic patients in Bangladesh. On the other hand, a study in Calcutta revealed a very low prevalence of STEC among hospitalized patients with diarrhea (*i.e*., 1.4% from bloody and 0.6% from watery stool samples), as reported by Khan *et al*. (2002). Another study in India also found no STEC in children with diarrhea in Delhi (Bhan *et al*., 1989). Although slight variation in the prevalence of *E. coli* in our study was found as compared to some other studies; this variation might be due to difference in the patterns of study, for example, in the cases of those studies, samples were collected from people of different communities, age, sex, food habit and sometimes varying in religion. On the other hand, in our study, samples were collected only from the hospitalized diarrheic children aging between 0-5 years of age.

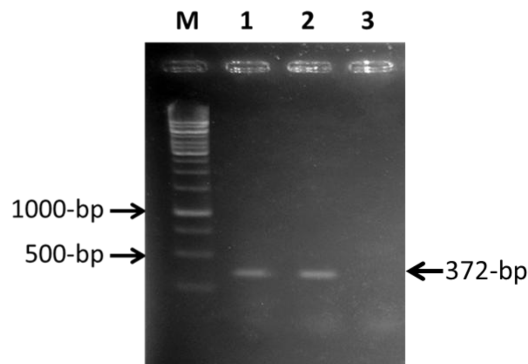

Figure 2. Amplification of *Stx*-2 (372 bp) genes; Lane 1: 1 kbp DNA ladder, Lane 2: *Stx*-2 positive gene from *E. coli*; Lane 3: Positive control; Lane 4: Negative control

Table 3. Antibiogram profile of *E. coli* isolates

Antimicrobial agents and concentration (μg)		No. of isolates (%)		
		R	I	S
Amoxycillin	30	24 (88.88)	3 (11.11)	0 (0.0)
Azithromycin	30	23 (85.18)	4 (14.81)	0 (0.0)
Ciprofloxacin	5	8 (29.62)	4 (14.81)	13 (48.14)
Cefixime	30	17(62.96)	2 (7.40)	8 (29.63)
Ceftriaxone	30	19 (70.37)	0 (0.0)	8 (29.63)
Cephradine	5	23 (85.18)	2 (25)	4 (14.81)
Gentamicin	10	2 (7.40)	-	25 (92.59)
Levofloxacin	5	19 (70.37)	0 (0.0)	8 (29.63)
Moxifloxacin	5	16 (59.25)	2 (7.40)	9 (33.33)

*R= Resistant: I=Intermediate: S = Sensitive

Table 4. Frequency of distribution of multidrug resistant (MDR) *E. coli* isolates from collected samples

Resistance profiles	*E. coli*
	No. of isolates (%)
No resistance demonstrated	-
Resistant to 2 agent (AMX-AZM)	6(22.23)
Resistant to 3 agents	
• AMX-AZM–CFM	1(3.70)
• AMX-AZM-CH	1(3.70)
Resistant to 4 agents	
• AMX-AZM-CFM-CIP	2(7.40)
• AMX-AZM-CFM-LE	2(7.40)
• AMX-AZM-CFM-CH	2(7.40)
• AZM-LE-CFM-CTR	1(3.70)
Resistant to 5 agents	
• AMX-LE-CFM-CH-CTR	3(11.11)
• AMX-CIP-CH-GEN-LE	1(3.70)
Resistant to 6 agents	
• AMX-AZM -LE-CFM-CTR-MOX	3(11.11)
• AZM-AMX-GEN-CFM-CTR-CH	2(7.40)
• AZM-AMX-GEN-CFM-MOX-CH	2(7.40)
Resistant to 7 agents	
• AMX-AZM-CFM—CIP-CH-LE-MOX	1(3.70)
Total resistant isolates	27(100)

Over the last few decades, STEC has been found to be the main cause of diarrheal infection manifested by watery to severe bloody diarrhea in human. Bangladesh is also considered as an important endemic area for diarrheal diseases. Previous report showed that more than 5% of children aging less than 5 years were attributed to diarrhea every year in Bangladesh (Arifeen *et al*., 2005). In our study, the prevalence of STEC in children was 1.20%, supported the findings of Arifeen *et al*. (2005) and Rehman *et al*. 2014). However, the causes behind the low prevalence of STEC associated diarrhea are not properly understood in Bangladesh (Islam *et al*., 2006) and India (Khan *et al*., 2002).

In this study, characteristics colonies of *E. coli* were observed on EMB agar, MC agar, which was similar to the findings of several previous reports (Nazir *et al*., 2005; Nazir, 2007; Hassan *et al*., 2014; Mamun *et al*., 2016; Tanzin *et al*., 2016). In Gram's staining method, the isolated bacteria exhibited pink, small rod shaped Gram-negative bacilli. These findings were in support of the findings of Nazir *et al*. (2005) and Islam *et al*. (2016).

Stool isolates revealed a complete fermentation of basic sugars as stated by Mckec *et al*. *(*1995). *E. coli* isolates were able to ferment the five basic sugars producing both acid and gas; however, differentiation of *E. coli* into species level was difficult as showed similar reaction in various sugars. All the isolates fermented dextrose, sucrose, fructose, maltose and mannitol with the production of acid and gas within 24 h of incubation. Results of *E. coli* isolates were positive as reported by and Mamun *et al*. (2016). The isolates also revealed positive reaction in MR test and Indole test but negative reaction in V-P test, which was supported by several authors (Nazir et al., 2005; Zinnah *et al*., 2007; Abbas *et al*., 2015).

The antibiogram study of all of the 27 isolates against 9 antibiotics used in this study revealed that most of the isolates were MDR. More than 77.78% of the isolates were found to be resistant to at least three antibiotics. This finding was varied from the findings of Talukdar *et al*. (2013) who reported the rate as 36%. This variation might be due to use of old antibiotics in their studies, whereas few newer antibiotics were included in our study. Highest resistant pattern was shown against Amoxycillin (88.88%), Azithromycin (85.18%) and Cephradine (85.18%) followed by Ceftriaxone and Levofloxacin (70.37%) and Cefixime (62.96%). Ansari *et al.* (2014) reported 100% resistant pattern against Amoxycillin, whereas Islam *et al.* (2015) and Rehman *et al*. (2014) reported that 59.15% and 75% *E. coli* were resistant to Amoxycillin. Resistant pattern against Azithromycin (85.18%) found in this study was supported by Hossain *et al*. (2012).

In our study, highest sensitivity was found against Gentamicin (92.59%), as reported by Ansari *et al*. (2014) and Hossain *et al.* (2012). However Malik *et al*. (2013) revealed 51.21% sensitivity to Gentamycin. This variation might be due to extensive use of Gentamycin that caused to emergence of Gentamycin resistant *E. coli*. We also found that Ciprofloxacin was sensitive to 48.14% samples, which was supported by Dhanashree and Mallya (2008).

From the findings of the study, it may be concluded that, molecular confirmation of 27 (32.53%) isolates of *E. coli* out of 83 samples by PCR using 16SrRNA primer was performed. One isolate (1.20%) of *E. coli* was found as virulent using *Stx*-1 and *Stx*-2 primers by molecular technique (duplex-PCR). Gentamicin (92.59%) and Ciproflxacin (48.14%) are the most sensitive against isolated *E. coli.* So, these antibiotics can be recommended as the effective drugs against STEC infections in children.

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