

Full Length Research Paper

## Bovine Shiga toxin producing *Escherichia coli* O157:H7 of Bangladesh: Is it capable of causing diseases similar to clinical strains?

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*Escherichia coli* O157:H7 is a predominant serotype of Shiga toxin producing *E. coli* (STEC) and is responsible for many outbreaks worldwide. Until recently, there is no comparative study on the bovine and clinical isolates as no STEC O157:H7 has been isolated from patients in Bangladesh. In the present study, the local bovine isolates were compared with a reference clinical strain to investigate whether the bovine isolates are capable of producing same degree of illness as the clinical strain. Two local isolates (CD-11 and CD-17) of bovine origin and a reference clinical strain (*E. coli* O157:H7 NCTC 12079) were investigated for the presence of virulence genes by polymerase chain reaction (PCR), Shiga toxin production by VTEC-RPLA, invasive property by Congo red binding and Sereny's keratoconjunctivitis, and other enterotoxic, cytotoxic and mouse lethal activities. Both the local isolates and the reference clinical strain showed the presence of *eae* and *stx*<sub>2</sub> genes and were found to be non-invasive. The isolates also produced enterotoxin, cytotoxin and mouse lethality similar to that found with the reference strain. All these results strongly suggest that the local bovine STEC O157:H7 isolates have potential to cause diseases similar to clinical STEC O157:H7 strain, which might lead to any outbreak in Bangladesh.

**Key words:** Bovine, *Escherichia coli*, STEC O157:H7, Bangladesh.

### INTRODUCTION

The bacterium *Escherichia coli* O157:H7 has been reported as the predominant serotype of Shiga toxin producing *E. coli* (STEC) (Armstrong et al., 1996; Besser et al., 1999; Tarr et al., 2005). Cattles are considered to be the principal natural reservoirs of the organisms, excreting the bacteria in their feces (Gansheroff and O'Brien, 2000; Molina et al., 2003). Consumption of foods, particularly undercooked ground beef and raw milk has been associated with large food poisoning outbreaks, in which this organism was identified as the etiologic agent (WHO, 1997). The first outbreak of STEC O157:H7

was recorded in the United States in 1982 and other outbreaks occurred later in the United Kingdom, continental Europe, Africa, New Zealand and Japan over the next decade (CDC, 1982; Coombes et al., 2011; Pennington, 2010). STEC O157:H7 infections cause hemorrhagic colitis and hemolytic uremic syndrome (HUS), which includes thrombocytopenia and acute renal failure.

The pathogenicity of STEC O157:H7 is associated with various virulence factors, such as Shiga toxins 1 and 2 (Stx1 and Stx2), that are encoded by *stx*<sub>1</sub> and *stx*<sub>2</sub> genes,

respectively. Stx1 is antigenically similar to Shiga enterotoxin produced by *Shigella dysenteriae* type 1. Stx2 is heterogeneous (Stx2c, Stx2d, Stx2e and Stx2f) and immunologically different from Stx1 (Nakao and Takeda, 2000). Shiga toxins are A<sub>1</sub>B<sub>5</sub> toxins that halt protein synthesis in the host cells, a process that may lead to an apoptotic cell death, and cause the vascular endothelial damage observed in patients with hemorrhagic colitis and the HUS (Paton and Paton, 1998). Another virulence factor is the protein intimin (encoded by the *eae* gene) which is responsible for the intimate attachment of the bacterium to the intestinal epithelial cells and causes the formation of attaching and effacing (A/E) lesions in the intestinal mucosa (Kaper et al., 1998). On the other hand, Stx exhibits cytotoxic effects on Vero and other cell lines. Hence, Stx is known as verotoxin (Vtx) and STEC are synonymously called verotoxin producing *E. coli* (VTEC). Stx is also a potent enterotoxin which is demonstrated by its ability to provoke fluid accumulation in rabbit ileal loops and is probably responsible for causing diarrhea (Blanco et al., 1991; Nataro and Kaper, 1998; Ferreira et al., 2002).

A number of studies were performed in different countries to compare the virulence factors of bovine and human STEC O157:H7 strains. Comparative studies in Ireland, Sweden and Japan revealed that cattle isolates possessing same phenotypic and genotypic traits as human clinical isolates have potential for causing human disease (Lenahan et al., 2009; Aspan and Eriksson, 2010; Lee et al., 2011). Kim et al. (1999) suggested that *E. coli* O157:H7 strains isolated from diseased humans were members of a different lineage than strains typically isolated from healthy cattle. Bono et al. (2012) reported that cattle harbor one lineage of STEC O157:H7 subtypes which is rarely found in clinically ill humans. Thus, it might be possible that bovine STEC O157:H7 strains sharing many qualities with human clinical strains might not be associated with human diseases.

In Bangladesh, which is one of the developing countries, there is no report of any outbreak caused by the STEC O157:H7. In fact, most of the outbreaks were more often reported from the industrialized countries than from developing countries because of the advanced surveillance and reporting systems in the industrialized countries. However, there are few reports on the prevalence of the Shiga-toxin producing *E. coli* (STEC) in Bangladesh (Islam et al., 2007, 2008, 2010). All these reports included the isolation and molecular characterizations of these organisms from the diarrheal patients, slaughtered animals, raw meat and other food samples. Apart from these reports, no detail studies were reported describing the enteropathogenicity or virulence properties of the locally isolated STEC O157:H7 from bovine origin in Bangladesh. Also there is no comparative study on the bovine and clinical isolates as no STEC O157:H7 has been isolated from patients in the recent past in Bangladesh. Therefore, the question arises on how viru-

lent the bovine STEC O157:H7 isolates are or whether the bovine isolates are capable of producing same degree of illness as the clinical STEC O157:H7. In the present study, we tried to find out the answers to the above questions, where the virulence potential of the local bovine *E. coli* O157:H7 isolates was compared with a reference clinical strain, *E. coli* O157:H7 NCTC 12079.

## MATERIALS AND METHODS

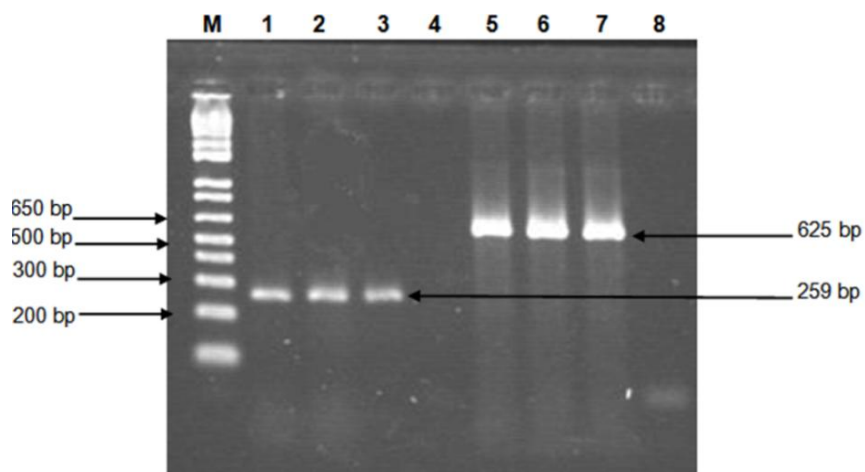
### Bacterial strains

A reference clinical strain *E. coli* O157:H7 NCTC 12079 and a negative control strain *E. coli* K-12 were obtained from the Department of Microbiology, University of Dhaka, Bangladesh. Two bovine STEC O157:H7 (CD-11 and CD-17) were isolated from the fresh feces of 18 healthy cattle from six different dairy farms around Dhaka city, Bangladesh and were included in the present study. The isolation and detection procedures of the bovine strains are described below:

One gram of each bovine feces sample was placed in 9 mL of trypticase soy broth (TSB) supplemented with 20 mg/mL novobiocin (Wako, Japan), and incubated at 37°C for 16-18 h. The enriched samples were streaked onto sorbitol MacConkey agar (Oxoid, England) plates supplemented with 0.5 mg/L cefixime and 1.5 mg/L potassium tellurite (Sigma, Germany) and incubated as above. After incubation, non-sorbitol fermenting colonies were streaked onto eosine methylene blue (EMB) agar (Oxoid, England) and 4-methylumbelliferyl-β-D-glucuronide (MUG) agar (Difco, USA) and incubated at 37°C for 18-22 h. Colonies showing green metallic sheen on EMB agar and no fluorescence on MUG agar, were characterized by indole production, citrate utilization, methyl red, Voges-Proskauer, triple sugar iron and oxidase tests as described by Cappuccino and Sherman (2011). Presence of the O157 and H7 antigens in biochemically positive colonies were investigated by latex agglutination test kit (Wellcolex™, Remel, USA) and the two STEC O157:H7 strains (CD-11 and CD-17) were re-confirmed by PCR targeting the *rfb*<sub>O157</sub> and *fliC*<sub>H7</sub> genes (Figure 1) (Paton and Paton, 1998; Gannon et al., 1997). The description of each primer pair is given in Table 1.

### Molecular detection of virulence genes by PCR

PCR was performed to detect the major known virulence genes *eae*, *stx*<sub>1</sub>, and *stx*<sub>2</sub> according to the previous studies (Kawasaki et al., 2005; Vidal et al., 2004). The details of the nucleotide sequence and size of the PCR amplicon for each primer pair are listed in Table 1. Template DNA was prepared by boiling DNA method as described by Radu et al. (2000). One bacterial colony was suspended in 1 ml of distilled water and boiled in a water bath for 10 min. After centrifugation at 10,000 rpm for 5 min, the supernatant was used as template DNA. PCR assay was performed using a thermal cycler (Bio-Rad, USA) in a total volume of 25 μL containing 2 μL of template DNA and 23 μL of master mix composed of 1 x PCR buffer (Takara, Japan), 0.5 μM (each) primer set, 0.2 mM each of the four deoxynucleotide triphosphates (dNTP mixture, Takara, Japan) and 0.5 U of TaKaRa Ex Taq™ polymerase (Takara, Japan). Chromosomal DNA of *E. coli* K-12 was used as negative control. The optimized PCR conditions for the genes assayed are shown in Table 2. The PCR amplicons were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and photographed using a gel documentation machine (Bio-Rad, USA). DNA markers (Invitrogen, USA) were used as size references.



**Figure 1.** Agarose gel electrophoresis showing 259 and 625 bp PCR amplification products of *rfb*<sub>O157</sub> and *fliC*<sub>H7</sub> genes respectively. M = 1 kb plus DNA ladder; 1, 5 = CD-11; 2, 6 = CD-17; 3, 7 = *E. coli* O157:H7 NCTC 12079; 4, 8 = *E. coli* K-12.

**Table 1.** Primers used in the study.

Target gene	Primer name	Primer sequence (5'→3')	Amplicon size (bp)	Reference
<i>rfb</i> <sub>O157</sub>	O157F	CGGACATCCATGTGATATAGG	259	Paton and Paton (1998)
	O157R	TTGCCTSTGTACAGCTAATCC		
<i>fliC</i> <sub>H7</sub>	FLICH7-F	GCGCTGTCGAGTTCTATCGAG	625	Gannon et al. (1997)
	FLICH7-R	CAACGGTGACTTTATCGCCATTCC		
<i>eae</i>	VS8	GGCGGATTAGACTTCGGCTA	150	Kawasaki et al. (2005)
	VS9	CGTTTTGCCACTATTGCC		
<i>stx</i> <sub>1</sub>	LP30	CAGTTAATGTGGTGGCGAAGG	348	Vidal et al. (2004)
	LP31	CACCAGACAAATGTAACCGCTC		
<i>stx</i> <sub>2</sub>	LP41	ATCCTATTCCCGGGAGTTTACG	584	Vidal et al. (2004)
	LP42	GCGTCATCGTATACACAGGAGC		

**Table 2.** PCR conditions used for the detection of various genes of STEC O157:H7.

Stage	PCR assay			
	<i>rfb</i> <sub>O157</sub>	<i>fliC</i> <sub>H7</sub>	<i>eae</i>	<i>stx</i> <sub>1</sub> and <i>stx</i> <sub>2</sub>
Initial denaturing	94°C: 10 min	94°C: 10 min	94°C: 10 min	94°C: 10 min
Denaturing	94°C: 1 min	94°C: 30 s	94°C: 20 s	94°C: 1 min
Annealing	56°C: 1 min	65°C: 30 s	60°C: 30 s	55°C: 1 min
Extension	72°C: 1 min	72°C: 75 s	72°C: 30 s	72°C: 1 min
Cycle no.	35 cycles	35 cycles	35 cycles	35 cycles
Final extension	72°C: 7 min	72°C: 7 min	72°C: 7 min	72°C: 7 min

**Detection of Shiga toxin production**

The types of Shiga toxin produced by each isolate of STEC O157:H7 were determined using VTEC-Reverse Passive Latex Agglutination (RPLA) test kit (Oxoid TD960) following the procedure described in the kit manual.

**Congo red binding test**

Trypticase soy agar containing 0.01% (w/v) Congo red was streaked with both bovine and reference clinical strains and incubated at 37°C for 18 h. After incubation, colonies were examined for the presence (colonies with dark red center) or absence

(colonies without dark red center) of Congo red binding (Sharma et al., 2006).

#### Animal maintenance

All the animal experiments were undertaken following the ethical issues set the Faculty of Biological Sciences, University of Dhaka (Reference number 5210). All efforts were made to minimize the sufferings of the animals.

#### Sereny's keratoconjunctivitis test

This test was performed according to the procedure as reported previously (Sereny, 1955). Briefly, 20  $\mu$ L of an overnight culture of the STEC O157:H7 strains (both bovine isolates and reference clinical) or *Shigella flexneri* 2a containing approximately  $5 \times 10^8$  cells/ml in phosphate buffer saline (PBS) was inoculated into one of the eyes of a guinea pig and the other eye served as the negative control. A total of four guinea pigs were included in each group and the *S. flexneri* 2a inoculated eyes served as the positive control. All guinea pigs were observed daily for 5 days for any inflammatory responses in the eyes of the animals.

#### Preparation of live cells

Ten milliliters of Brain Heart Infusion (BHI) broth was inoculated with 5 colonies of the pure culture of each strain (bovine or reference clinical) and incubated at 37°C for 6 h with shaking. One milliliter BHI broth containing approximately  $10^5$ - $10^6$  cfu was used as inoculum for the enterotoxicity assay (Sanyal et al., 1975).

#### Preparation of culture filtrate

Fifty milliliters conical flasks containing 10 ml of BHI broth were inoculated with 5 colonies of the pure cultures of each strain (bovine isolates or reference clinical) and incubated at 37°C for 20 h with shaking (100 rpm). The cultures were centrifuged at 10,000 rpm for 10 min at 10°C and each supernatant was filtered through a Millipore membrane (0.45  $\mu$ m pore diameter) and preserved at -20°C. These culture filtrates were used for enterotoxicity, cytotoxicity and mouse lethality assays.

#### Enterotoxicity assay

Detection of enterotoxic ability of Stx produced by the STEC O157:H7 strains was tested in the rabbit ileal loop (RIL) following the procedure of Sanyal et al. (1975). One milliliter each of a 6 h grown bacterial cultures or the culture filtrates prepared above, were tested in loops of an adult rabbit ileum (New Zealand White variety, weighing 1.8-2.0 kg). Each sample was tested in duplicate rabbits following laparotomy and live cells or culture filtrate of *Vibrio cholerae* 569B and the culture filtrate of *E. coli* K-12 or the BHI broth were inoculated as positive and negative controls, respectively. The inoculated rabbits were euthanized after 18 h and the volume of fluid accumulation per cm of gut in each rabbit was measured. Fluid accumulation  $\geq 0.5$  ml/cm was considered as positive.

#### Cytotoxicity assay

Cytotoxicity of the STEC O157:H7 culture filtrates were tested on HeLa and MDCK (Medin-Derby canine kidney) cells (Konowalchuk

et al., 1977). HeLa or MDCK cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Freshly trypsinized cells were counted, suspended in growth medium (approximately  $1 \times 10^5$  cells/ml) and distributed in 0.1 ml volume into 96-well cell culture plates. Monolayers of cells were established by 18 to 20 h of incubation at 35°C in a 5% CO<sub>2</sub> atmosphere and 0.1 ml volume of each culture filtrate was added to the cell monolayers. The plates were incubated for an additional 18 to 20 h and checked for any change in cell morphology. BHI medium was used as negative control and each sample was checked in duplicate wells.

#### Mouse lethality assay

Five groups of Swiss Albino mice, each comprising of six animals, were injected intraperitoneally with the culture filtrates (0.1 ml each) of the STEC O157:H7 strains (Tesh et al., 1993). Negative control animals received the *E. coli* K-12 culture filtrate or BHI broth only. All mice were kept in cages with free access to food and sterile water, monitored daily for 10 days and any change in behavior or sickness was recorded.

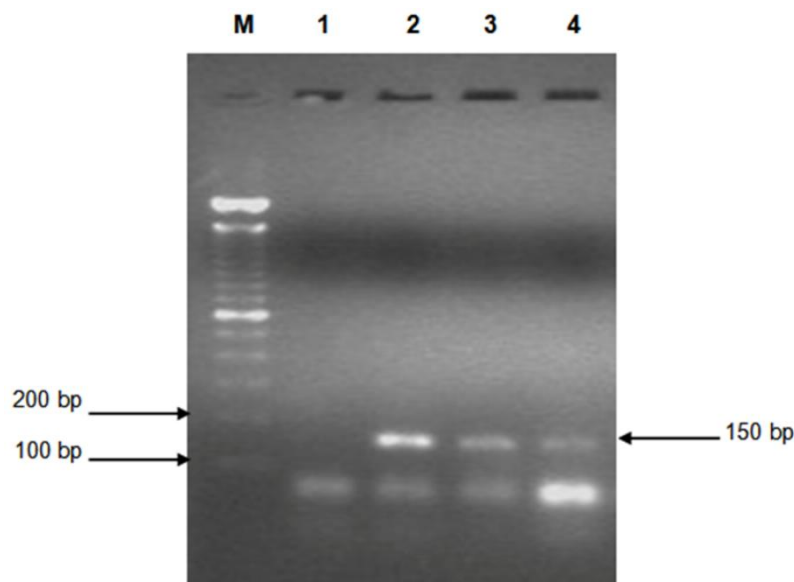
## RESULTS AND DISCUSSION

Shiga toxin producing *E. coli* (STEC) are food-borne pathogens that cause hemolytic colitis and a serious sequel, HUS. The largest outbreaks of STEC are due to a single *E. coli* serotype, O157:H7, although STEC non-O157:H7 serotypes also cause similar diseases (Khan et al., 2002). There are a number of comparative studies between human disease and bovine-associated STEC O157 in different countries. A study on cattle and human clinical isolates of STEC O157 showed that cattle isolates possessed all the virulence traits that are typical for the human pathogenic strains. On the other hand, the human clinical isolates had different combinations of Stx-variants as compared to the cattle isolates (Nielsen and Scheutz, 2002). In another study in Czech Republic, it was reported that the phenotypic and genotypic characteristics of both bovine and human STEC O157 isolates were identical or closely related which supported the pathogenic potential of the bovine isolate for humans (Bielaszewska et al., 2000). An investigation of STEC O157 strains isolated from human sporadic infections with those of cattle found that human clinical isolates constitute a small fraction of bovine isolates (Roldgaard et al., 2004). However, Baker et al. (2007) demonstrated that STEC O157:H7 isolates from healthy cattle were less virulent than those from human disease outbreaks. Bangladesh is an endemic zone for diarrheal diseases and in recent years, STEC non-O157:H7 has also been isolated from hospitalized patients. Although the STEC non-O157:H7 is an uncommon pathogen among the hospitalized patients with diarrhea, however, the low prevalence of STEC non-O157:H7 in Bangladesh might be due to acquired immunity against the pathogen in the population (Islam et al., 2007). Apart from the few reports on the isolation and virulence study of STEC O157:H7 from slaughtered animals, no STEC O157:H7 has been

**Table 3.** Detection of Congo red binding, Sereny’s test, enterotoxigenic activity, cytotoxic activity, mouse lethality assay and presence of virulence genes in the STEC O157:H7 strains.

STEC O157:H7	Congo red binding	Sereny’s test	Enterotoxigenic activity (ml/cm)	Cytotoxic activity	Mouse lethality assay	<i>eae</i> gene	<i>stx1</i> gene	<i>stx2</i> gene	Toxin detection by RPLA
CD-11	-	-	2.2	+	+	+	-	+	Stx 2
CD-17	-	-	2.0	+	+	+	-	+	Stx 2
NCTC 12079	-	-	2.4	+	+	+	+	+	Stx 1 and 2
<i>E. coli</i> K-12	ND	ND	-	-	-	-	-	-	-
<i>V. cholera</i> 569B	ND	ND	3.0	ND	ND	ND	ND	ND	ND

ND = not done; ‘+’ = positive result; ‘-’ = negative result.

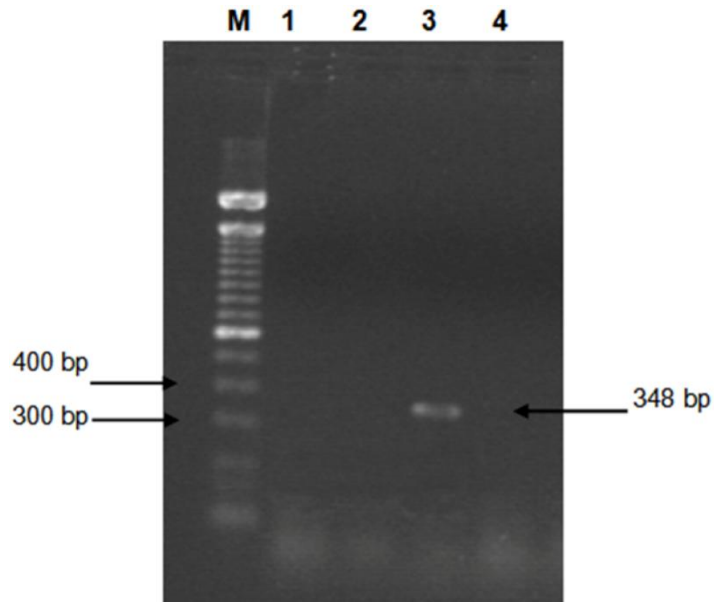


**Figure 2.** Agarose gel electrophoresis showing 150 bp PCR amplification products of *eae* gene. M = 100 bp DNA ladder; 1 = *E. coli* K-12; 2 = *E. coli* O157:H7 NCTC 12079; 3 = CD-11; 4= CD-17.

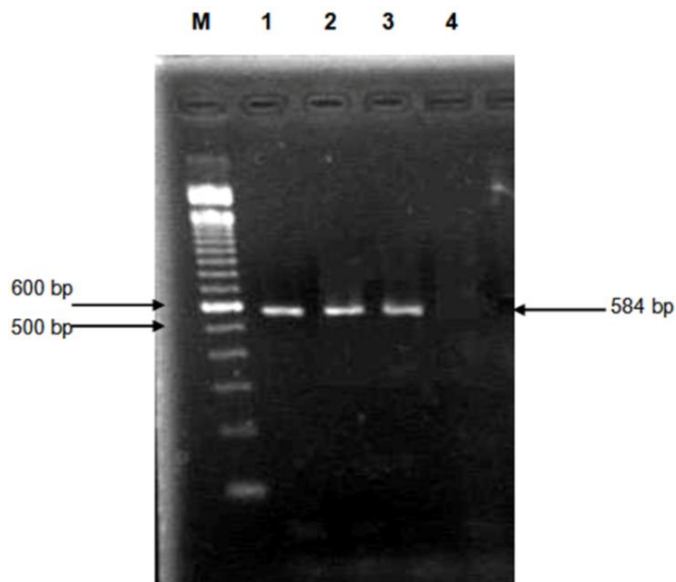
isolated either from diarrheal patients or other sources in Bangladesh (Islam et al., 2008). Therefore, not much is known about the virulence properties of this pathogen isolated either from the patients or the cattle. In the present study, the pathogenicity and virulence potential of two bovine STEC O157:H7 isolates were compared to that of a reference clinical *E. coli* O157:H7 NCTC 12079 strain to understand the virulence potential of the local bovine isolates.

Table 3 summarizes comparative virulence traits of local bovine isolates (CD-11 and CD-17) and the reference clinical strain *E. coli* O157:H7 NCTC 12079. PCR is generally considered to be the most sensitive means of determining whether a fecal specimen or a food sample contains STEC (Paton and Paton, 1998). In our PCR assay, the presence of the main virulence genes (*eae*, *stx1* and *stx2*), which have been widely used by

other researchers, were investigated and only the *stx2* gene was detected in both bovine isolates. On the other hand, both *stx1* and *stx2* genes were detected in the reference strain (Figures 2 to 4). It has been reported that the *stx2* gene was more common in bovine than the *stx1* in most of the studies performed in the USA, Japan and European countries (Ding et al., 2011). Epidemiologic data suggest that STEC O157 strains that express Stx2 are more important than Stx1 in the development of HUS and strains that express Stx2 alone are more likely to be associated with the progression to HUS than strains that produce both Stx1 and Stx2 (Griffin and Tauxe, 1991; Griffin, 1995). Numerous investigators have also shown strong association between the carriage of *eae* gene and the capacity of STEC causing severe human disease, especially HUS (Suardana et al., 2011). In this study, this important virulence gene was also detected by PCR in



**Figure 3.** Agarose gel electrophoresis showing 348 bp PCR amplification products of *stx*<sub>1</sub> gene. M = 100 bp DNA ladder; 1 = CD-11; 2 = CD-17; 3 = *E. coli* O157:H7 NCTC 12079; 4 = *E. coli* K-12.



**Figure 4.** Agarose gel electrophoresis showing 584 bp PCR amplification products of *stx*<sub>2</sub> gene. M = 100 bp DNA ladder; 1 = CD-11; 2 = CD-17; 3 = *E. coli* O157:H7 NCTC 12079; 4 = *E. coli* K-12.

both the STEC O157:H7 isolates and reference strain, which gave evidence that these strains had potency to colonize the intestine and induce attaching-effacing lesions and also cause cytopathic effects in intestinal epithelial cells. Beutin et al. (2004) reported that Shiga

toxin type 1c and 2d were found to be present only in *eae* negative STEC strains, and type 2 was significantly more frequent in *eae*-positive STEC strains. Heuvelink et al. (1998) reported that bovine strains of STEC O157 share virulence factors with human strains, including *stxs* and *eae*,

and may be considered potential human pathogens. Our isolates were also evaluated for Shiga toxin production through VTEC-RPLA and the results were 100% in concordance with PCR results.

In the Congo red binding test, all of the three STEC O157:H7 strains (both bovine and reference clinical) produced colourless (non-Congo red binding) colonies, which indicated that the strains were non-invasive (Uhlich et al., 2002). The Congo red binding test was again supported by the Sereny's test, where none of these three strains could produce keratoconjunctivitis in guinea pig eyes, indicating non-invasive nature of the strains. However, the positive control *S. flexneri* 2a strain showed fully developed keratoconjunctivitis with purulence in guinea pig eyes within three days.

In the ligated rabbit ileal loop assay, both live cells and culture filtrates prepared from the bovine isolates and the reference clinical strain, induced fluid accumulation in the large gut of rabbits almost in the same range as that of the positive control *V. cholerae* 569B strain (Table 3). This is probably due to the release of sufficient amount of enterotoxin produced by the live cells *in vivo* in the large gut or *in vitro* during the 20 h period of cultivation of the STEC strains. The fluid accumulation in the large gut also indicated the potential of the bovine isolates to produce diarrhea in the host system in the same range as the reference clinical strain. Again in the cytotoxicity assay, the culture filtrates prepared from the bovine strains changed the cell morphology of both HeLa and MDCK cells, indicating the cytotoxicity of the bovine STEC O157:H7 strains.

The degree of cytotoxicity of the bovine culture filtrates was the same with the reference clinical culture filtrate. Also in the mouse lethality assay, all mice which received culture filtrates prepared from the bovine or reference clinical STEC O157:H7 strain became sick in two days. All these animals were unable to move, probably due to paralysis of the hind legs and died between 3-6 days after the culture filtrate injection, which could be due to Shiga toxin production by both bovine and the reference clinical strains (Obata et al., 2008). However, the negative control animals were found to be in good health and survived the mouse lethal activity assay.

All these findings clearly indicate that the bovine STEC O157:H7 isolates, CD-11 and CD-17, are as virulent as the reference clinical STEC O157:H7 strain. In a recent report, it was found that STEC O157:H7 increased its pathogenicity in the animal model after the passage through the gastrointestinal tract of the same host (Fernandez-Brando et al., 2012). This is a matter of concern, as the bovine STEC strains, if passaged through a suitable host, might increase its pathogenicity. Although no one single animal model fully represents the spectrum of STEC illness, however, the results of the study strongly suggest that the bovine STEC O157:H7 isolates have potential to cause disease similar to the clinical STEC O157:H7 strains, which might also lead to

any future outbreak in Bangladesh. Further studies are required with large number of isolates from various sources for better understanding of the virulence potential of local STEC O157:H7. In addition, more virulence characteristics and clonal relatedness of isolates can be included in these studies.

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