J. Bangladesh Agril. Univ. 14(1): 63–68, 2016 ISSN 1810-3030

Prevalence and molecular detection of shiga toxin producing *Escherichia coli* from diarrheic cattle

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are zoonotically important pathogen which causes hemorrhagic colitis, diarrhea, and hemolytic uremic syndrome in animals and humans. The present study was designed to isolate and identify the STEC from fecal samples of diarrheic cattle. A total of 35 diarrheic fecal samples were collected from Bangladesh Agricultural University (BAU) Veterinary Teaching Hospital. The samples were primarily examined for the detection of *E. coli* by cultural, morphological and biochemical characteristics, followed by confirmation of the isolates by Polymerase Chain Reaction (PCR) using gene specific primers. Later, the STEC were identified among the isolated *E. coli* through detection of *Stx*-1 and *Stx*-2 genes using duplex PCR. Out of 35 samples, 25 (71.43%) isolates were confirmed to be associated with *E. coli*, of which only 7 (28%) isolates were shiga toxin producers, and all of them were positive for *Stx*-1. However, no *Stx*-2 positive isolate could be detected. From this study, it may be concluded that cattle can act as a reservoir of STEC which may transmit to human or other animals.

Keywords: Prevalence, STEC, Isolation, PCR, Importance

Introduction

Escherichia coli (*E. coli*) is a Gram-negative, rod-shaped, flagellated, motile, oxidase negative, facultative anerobic organism, and is classified under the family Enterobacterioceae (Riley *et al*., 1983). *E. coli* produces septicemia and diarrhea in cattle and other animals such as piglets, kids, foals and lambs. Cats and dogs are susceptible to cystitis and other urogenital infections caused by *E. coli* (Riley *et al*., 1983). Pathogenic *E. coli* are mainly divided into two types namely Enteropathogenic *E. coli* and uropathogenic *E. coli.* Further, pathogenic *E. coli* are grouped into enterotoxic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC) and enterohaemorrhagic *E. coli* (EHEC) (Riley *et al*., 1983). *E. coli* O157:H7 is an enterohemorrhagic strain of the bacterium *E. coli* and a cause of illness through food. While most strains are harmless and normally found in the intestines of mammals, this strain may produce shiga-like toxins which is a member of a class of pathogenic *E. coli* known as EHEC. Often they are referred to depending on their toxin producing capabilities, known as verocytotoxin producing *E. coli* (VTEC) or shiga-like toxin producing *E. coli* (STEC). The etiologic agent of the illness was identified as a rare O157:H7 serotype of *E. coli* in 1983. This serotype had only been isolated once before, from a sick patient in 1975 (Riley *et al*., 1983).

STEC is an important group of *E. coli* that can cause severe diarrhea and food-borne illness worldwide. The serotype of a STEC is based on the 'O' antigen determined by the polysaccharide portion of cell wall lipopolysaccharide and the 'H' antigen by the flagella protein. STEC can grow in temperatures ranging from 7°C to 50°C, with an optimum temperature of 37°C (Rehman *et al*., 2014). Healthy dairy and beef cattle are a major reservoir of STEC (Rehman *et al*., 2014). Contamination of meat with the bacterium can occur during slaughtering. Ground beef possesses higher risk than intact meat because it can be contaminated during the grinding process (Rehman *et al*., 2014). Besides, food and water can be contaminated by cattle manure. In many countries, STEC was isolated from diarrheic cattle as one of the major causal agents of diarrhea. In Bangladesh, the incidence of diarrheal diseases in calf is high. However, so far no report has been observed on the detection or isolation of STEC from diarrheic cattle in Bangladesh. The prevalence of *E. coli* infection was higher in diarrheic calves (13.71%) as compared to that of non-diarrheic calves (9.1%), as reported by Nazir *et al*. (2005). Considering the above points, the present study was conducted to isolate and detect shiga toxin producing *E. coli* from diarrheic cattle.

Materials and Methods

Collection of samples

A total of 35 fecal samples were collected from diarrheic cattle that were presented to the Bangladesh Agricultural University (BAU) Veterinary Teaching Hospital, Mymensingh during the period from January 2015 to March 2015. After collection, each sample was inoculated into nutrient broth (NB) and incubated at 37^0C overnight.

Isolation of *E. coli* in culture media

After primary culture of the organism, a 10 fold dilution was made to reduce overgrowth of the organisms. Then 100 µL was inoculated onto Mac-Conkey agar. Single well defined pink colony was further sub-cultured onto the Eosin Methylene Blue (EMB) agar media.

Gram staining

The *E. coli* colonies were characterized morphologically using Gram stain according to the method described by Nazir *et al*. (2005).

Identification by biochemical test

The sugar fermentation test was performed by inoculating a loop full of NB culture of the organisms into each tube containing five basic sugars (*e.g*., dextrose, sucrose, lactose, maltose, and mannitol) separately and incubated for 24 h at 37^0C. Indole test, MR-VP test were performed accordingly to the method described by Nazir *et al*. (2005).

Molecular Detection of *E. coli* by PCR

DNA extraction by boiling method

A pure bacterial colony was mixed with 200 uL of distilled water which were boiled for 10 min, then immediately kept on ice for cold shock followed by centrifugation at 10,000 rpm for 10 min. The supernatant was collected and used as DNA template in PCR.

Primers used for PCR

Oligonucleotide primers 5´GAC-CTC-GGT-TTA-GTT-CAC-AGA3´ (Forward) and 5´-CAC-ACG-CTG-ACG-CTG-ACC-A-3´ (Reverse) were used for the specific amplification of genus specific 16SrRNA gene of *E. coli* at 585-bp (Schippa *et al*., 2010). Similarly, primers 5´-CAC-AAT-CAG-GCG-TCG-CCA-GCG-CAC-TTG-CT-3´ (Forward) and 5´-TGT-TGC-AGG-GAT-CAG-TCG-TAC-GGG-GAT-GC-3´(Reverse) were used for EC*Stx*-1 at 606-bp (Heuvelink *et al.* 1995) and primers 5´-CCA-CAT-CGG-TGT-CTG-TTA-TTA-ACC-ACA-CC-3´ (Forward) and 5´-GCA-GAA-CTG-CTC-TGG-ATG-CAT-CTC-TGG-TC-3´ (Reverse) were used for EC*Stx*-2 at 374-bp (Heuvelink *et al.*, 1995).

PCR Assay

A total volume of 25 µL of PCR reaction mixture was prepared; the mixture contained PCR master mixture (Promega, USA): 12.5 µL, Forward primer (10 pmol/µL): 1 µL, Reverse primer (10 pmol/µL): 1 µL, DNA template: 5 µL, Nucleus free water: up to 25 µL. Thermal profiles for the amplification of 16SrRNA gene were 95^0C for 5 min in initial denaturation, 94^0C for 30 sec in denaturation, 58^0C for 1 min in annealing, 72^0C for 1 min in extension, 72^0C for 10 min in final extension and holding temperature was 4^0C until use. The total cycling was 30 times. Similarly, thermal profile to amplify *Stx*-1 and *Stx*-2 genes of *E. coli* were 95^0C for 5 min, followed by denaturation at 94^0C for 30 sec, 56^0C for 1 min in annealing, 72^0C for 1 min in extension, and 72^0C for 10 min for final extension and holding temperature was 4^0C until use. The total cycling was 30 times.

Agarose gel electrophoresis of PCR products

PCR products were analyzed by 1.5% agarose gel electrophoresis. In brief, gel containing tray was assembled with gel comb of appropriate teeth size and number. 1.5% Agarose gel was prepared in TAE buffer by boiling in a microwave over followed by solidification and preparing gel cast. The PCR product of each sample was electrophoresed at 100 V for 45 min and stained with ethidium bromide (EtBr) for 10 min. After that the PCR products were analyzed by trans-illuminator (biometra, Germany) and photograph was taken.

Results and Discussion

Cultural properties of *E. coli* on Mac-Conkey and EMB agar

E. coli was detected in 25 out of 35 samples. They were gram-negative, pink colored, small rod shaped, single or paired organisms but produce bright pink colonies on Mac-Conkey agar and greenish black colonies with metallic sheen on EMB agar. The media used in this study were selected considering the experience of the past researchers worked in various fields relevant to the present study (Nazir *et al*., 2005; Nazir, 2007). Colony growth character on different media exhibited characteristics reaction. In this study, colony characteristics of *E. coli* observed on EMB and MC agars were similar to the findings of other authors (Rehman *et al*., 2014; Hassan *et al*., 2014; Mamun *et al*., 2016; Tanzin *et al*., 2016; Elafify *et al*., 2016; Islam *et al*., 2016).

Identification of *E. coli* by Gram staining

The microscopic examination of isolated bacteria with Gram stained smears from Mac-Conkey and EMB agar revealed that the isolated bacteria were Gram negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain. These findings were in support of the findings of Hassan *et al*. (2014), Tanzin *et al*. (2016), Elafify *et al*. (2016) and Islam *et al*. (2016).

Identification of *E. coli* by Biochemical tests

In fermentation test, all the isolates fermented the five basic sugars (dextrose, sucrose, lactose, maltose and manitol) and sorbitol producing both acid and gas. Acid production was indicated by the color change from reddish to yellow and the gas production was noted by the appearance of gas bubbles in the inverted Durham's tubes. This result was also reported by Nazir *et al*. (2005) and Rehman *et al.* (2014). Fecal isolates revealed a complete fermentation of basic sugars as stated by Beutin *et al. (*1993), Sandhu *et al.* (1996) and 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 (Elafify *et al*., 2016). 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 Nazir *et al.* (2005). Each isolate produced a dark red color in the reagent and bright red colony after incubation of methyl-red (MR) broth positive and no color was produced in Voges-Proskauer (VP) broth, indole positive. This indicated the isolates were *E. coli.* The isolates also revealed positive reaction in MR test and Indole test but negative reaction in VP test, which was supported by several authors (Hassan *et al*., 2014; Mamun *et al*., 2016; Tanzin *et al*., 2016; Elafify *et al*., 2016; Islam *et al*., 2016).

Confirmation of *E. coli* by PCR

PCR was performed to detect shiga toxin (*Stx*-1 and *Stx*-2) of *E. coli.* First of all, EC16SrRNA primer was used for the confirmation of *E. coli* sample. All 25 *E. coli* isolates were identified as *E. coli* sample and band of EC16SrRNA (585-bp) was formed in agarose gel. Primers targeting *Stx*-1 gene of *E. coli* amplified 606-bp fragments of DNA confirmed the identity of *Stx*-1 from EC16SrRNA positive *E. coli.* Finally, among 25 of *E. coli* isolates, only 7 isolates were found positive for *Stx*-1 confirmed by amplification of *Stx*-1 gene and band of *Stx*-1 (606-bp) was formed in agarose gel, and none of them was EC *Stx*-2 positive. The result of PCR presented in Fig. 1 (EC16SrRNA) and Fig. 2 (EC*Stx*-1).

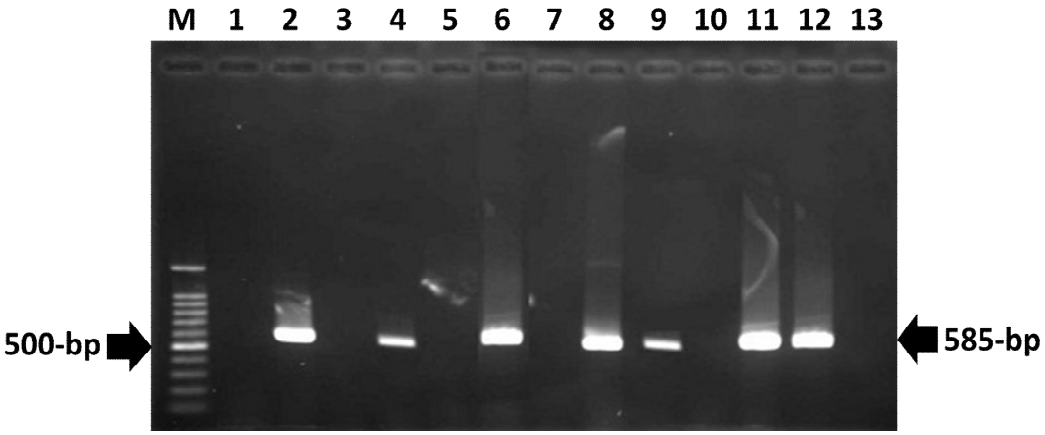

Fig. 1. PCR of *E. coli* specific 16SrRNA (585-bp); Lane M: 100-bp DNA ladder, Lane 2,4,6,8,9,11: positive for EC16SrRNA; Lane 12: Positive control for EC16SrRNA; Lane 13: Negative control.

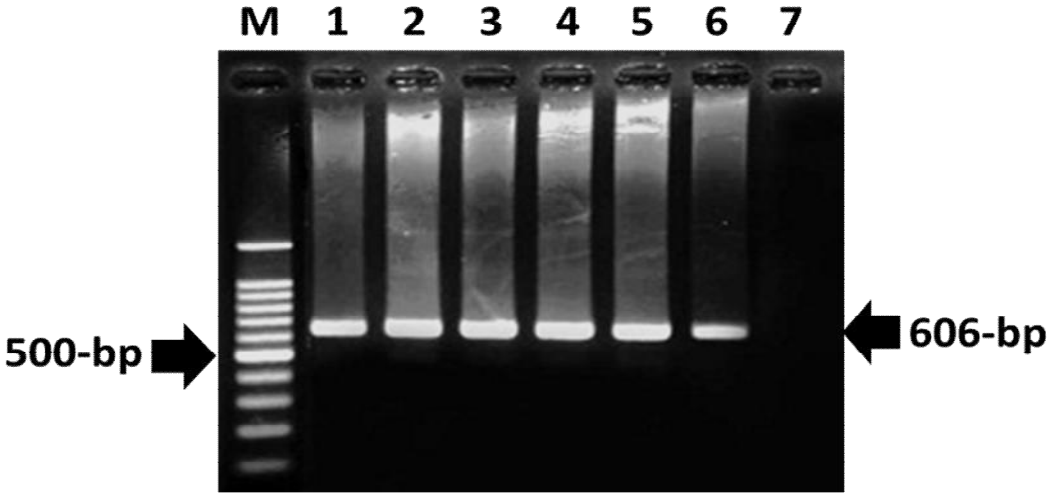

Fig. 2. Amplification of shiga toxin producing genes (*Stx*-1 and *Stx*-2) by multiplex PCR; Lane M: 100 bp DNA ladder, Lane 1-5: EC *Stx*-1 positive; Lane 6: Positive control for *Stx-1*; Lane 7: Negative control.

Prevalence of shiga-toxin producing *E. coli*

The prevalence of STEC in the isolated *E. coli* was 71.43% (n=25/35). Out of 25 *E. coli* isolates 7 samples were confirmed as STEC due to presence of shiga-toxin producing gene *Stx*-1, no *Stx*-2 positive *E. coli* was detected in this study.

Table. 1 Prevalence of shiga toxin producing *E. coli*

No. of samples	*E. coli* on the basis of cultural and biochemical properties	Sample positive for *E. coli* by PCR		Samples positive for STEC of the isolates *E. coli* by multiplex PCR	
		No.	%	No.	%
35	25	25	**71.43***	7	**28***

*p<0.01 (Significant)

In this study, out of 35 samples, 25 were confirmed to be associated with *E. coli* and out of these 25 isolates, only 7 were shiga-toxin producers, and all of them were positive for *Stx*-1. No *Stx*-2 positive isolates was detected in duplex PCR. This result suggested that 28% (p<0.01) isolates were bearing*Stx*-1 gene. From this study, it is concluded that 71.43% (p<0.01) cattle were positive for *E. coli* and 28% (p<0.01) were positive for STEC.

In present study, the results of PCR showed that 28% (p<0.01) stains carried *Stx*-1 gene and all the strains were *Stx*-2 negative, 7 (28%) were positive for *Stx*-1 genes. Higher frequency of *Stx*-1 gene (28%) than *Stx*-2 gene (0%) in diarrheic cattle observed in the present study is contrary to the observations of Dastmalchi *et al. (*2012) who reported predominance of *Stx*-2 over *Stx*-1 in diarrheic calves in Iran. This variation might be due the difference in geographical location, management practices and immune status of the animals.

The prevalence of STEC, including STEC O157, was significantly higher in buffalo feces than in cow or goat feces (p<0.05). Within similar geographical locations, a few studies of the occurrence of STEC have been done, and most of the studies were done in India and Thailand (Leelaporn *et al.,* 2003*;* Manna *et al.,* 2006). Recently, a study was done in central Vietnam that found a prevalence of STEC of 27% in buffaloes, 23% in cattle, and 38.5% in goats. In India, Manna *et al.,* (2006) reported that the prevalence of STEC O157 in fecal samples from slaughtered cattle and diarrheic calves was 2.0% and 7.6%, respectively (Manna *et al.,* 2006).

STEC O157 has also been isolated in India from foods of cattle origin namely raw minced beef samples (9%; n=22) (Dutta *et al.,* 2000), beef surface swabs (3.7%; n=27), and milk samples (2.4%; n=81) (Manna *et al.,* 2006). In China, STEC O157:H7 was isolated from 10 to 20% of the animals in the villages, including pigs, cattle, goats, and chickens (Zheng *et al.,* 2005; Fernández *et al*., 2009). When buffalo samples were tested for the presence of *Stx1* genes by PCR, around 80% of the samples were positive. Of the cows and goats, around 73% and 12%, respectively, were positive. By observing that prevalence rate of different animals we collected fecal sample from diarrheic cattle for the detection of *Stx*-1 and *Stx*-2 genes and only *Stx*-1 gene was amplified from 7 samples.

It is therefore difficult to determine whether the results reported reflect true differences in isolation rates or are the consequence of the different methodologies adopted. The relative occurrence of STEC virulence factors changed as the calves became older; as a result, *Stx-1* positive isolates might be replaced by *Stx*-2 positive isolates. In the current study, however, *Stx*-1 sequences were present in a higher number (28%) of isolates from diarrheic cattle. This may be due to differences in age of calves, season of collection, ruminal development, immune response, diet and other aspects of calves' management.

Conclusion

It is concluded that cattle are an important reservoir of virulent *E. coli* carrying toxigenic gene, which may cause illness in cattle as well as human.

Acknowledgement

The authors are grateful to National Science and Technology, Bangladesh for financial support.

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