

Original Article

Prevalence and characteristics of Shiga-toxin producing *Escherichia coli* (STEC) isolated from beef slaughterhouse

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ABSTRACT

Objective: Shiga-toxin producing *Escherichia coli* (STEC) is the most important foodborne bacterial pathogen worldwide and the bovine animals are assumed as a reservoir of this pathogen. The present study was conducted to assess the role of bovine animals as the source of STEC.

Materials and methods: To assess the role of bovine animals as the source of STEC, we examined 100 samples (50 rectal swab and 50 beef samples) collected from the local beef slaughterhouses by cultural, morphological, biochemical tests and polymerase chain reaction. Finally, the drug resistance pattern of isolated organisms has been examined.

Result: In the preliminary screening by polymerase chain reaction (PCR), *E. coli* was more prevalent in rectal swab (n=21/50) than beef samples (n=16/50). Among 39 isolated *E. coli*, 10 isolates were confirmed as STEC (Rectal swab=7, Beef=3) by PCR, where *stx2* gene (n=7/10) was predominant than *stx1* gene (n=3/10). Remaining 29 isolates did not react to *stx* primers in PCR. Presence of STEC in beef samples was significantly associated with the fecal contamination at $P \leq 0.1$ (0.074818) in Pearson's correlation coefficient method. In addition, most of the isolated STEC strains were resistant to one or more commonly used antimicrobials in the country.

Conclusion: The bovine animals and its products could be an important source of multidrug-resistant STEC in the country.

KEYWORDS

E. coli; Shiga-toxin; PCR; *stx1*; *stx2*

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INTRODUCTION

Escherichia coli is considered as the normal inhabitant of the gut microflora in mammals, however, in the exhausted or immunosuppressed host, nonpathogenic strains of *E. coli* can cause serious illness including diarrhea ([Duriez et al., 2001](#); [Hilbert et al., 2008](#)). Diarrheal illness is the causes of about 12600 deaths daily in children under 5 years of age in Asia, Africa and Latin America ([Nguyen et al., 2006](#)). Diarrheagenic *E. coli* (DEC) is the leading cause of foodborne diarrhea in developed as well as developing countries and this pathogen is an important etiology of morbidity and mortality worldwide ([Mohammed et al., 2002](#)).

In Bangladesh, about 34% of total diarrheal episodes are due to DEC ([icddr.b, 2002](#)) and this pathogen might be transmitted through foods of animal origin or water contaminated with human or animal feces. Person-to-person transmission may also take place ([Nataro and Kapler, 1998](#)). Unhygienic practices in food processing are mainly associated with food-borne diseases and diarrhea ([Wilfred et al., 2012](#)). The occurrences are generally related to food of bovine origin, minced beef and uncooked fresh milk ([Bachrouri et al., 2002](#)). Shiga-toxin producing *Escherichia coli* is worldwide reported DEC pathotype and is the causal agent of hemorrhagic colitis with some severe sequelae including hemolytic uremic syndrome, which is due to the effect of Shiga-toxin produced by the organism that acts on kidney, intestine and other parenchymatous organs ([Gyles, 2007](#)). STEC is characterized by the production of one or more types of Shiga-toxin (*stx1*, *stx2* or their variants), following cell death by inhibition of protein synthesis in host cells ([Strockbine et al., 1986](#)). Bovine animals are the natural reservoir of STEC contributing as the major source for human infections ([Dorn and Angrick, 1991](#)). The organism does not produce any clinical illness in their natural reservoir but it produces a broad spectrum of clinical abnormalities in humans including mild diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, bloody diarrhea and thrombotic thrombocytopenic purpura ([Fey et al., 2000](#)).

Harboring of STEC in cattle is a significant concern for public health because of their transmitting capability to humans through contaminated foods and water with feces from cattle ([Cooley et al., 2007](#)). Prevalence estimates ranging from 0.1 to 62% of *E. coli* in cattle was reported worldwide ([Pennington, 2010](#)). *E. coli* O157 is the best-known STEC pathogen until the date ([Caprioli et al., 2005](#)). The low infectious dose of the pathogen and

life-threatening complication has made this organism a serious threat to public health ([Akkaya et al., 2006](#)). In Bangladesh, the predominant pathotypes associated with childhood diarrhea is enterotoxigenic *E. coli* (ETEC) which accounts for 20% of all diarrheal cases in the country ([Qadri et al., 2005](#)). Recently, [Hassan et al. \(2017\)](#) reported 43.33% bovine animals in Bangladesh carry STEC in feces, which might be the source of STEC in Human. [Islam et al. \(2007\)](#) reported 0.5% prevalence of STEC among the hospitalized patients in Bangladesh and 1.9% among community patients. Routine monitoring of the slaughterhouses is crucial to producing safe food, as the animals are the potential source of the foodborne pathogen. Hence, the study was executed to elucidate the present status of STEC in local slaughterhouses and their drug-resistant pattern was investigated to find out whether the beef and its product could be a source of drug-resistant STEC.

MATERIALS AND METHODS

Sample collection: We collected 50 rectal swabs from cattle in five different slaughterhouses, after the animals were slaughtered following a convenience sampling method without repetition of animals. Sterile cotton buds were used for the collection of swab samples, and the swab was transferred to nutrient broth instantly. Meat samples (n=50) approximately weighing 50 gm were collected from the same slaughtered animal in sterile polyethylene sachets. The samples were transported on ice and processed immediately (within one hour).

Cultural and biochemical examinations: The nutrient broth containing swab samples have been incubated overnight at 37°C. After overnight incubation, enriched samples from the nutrient broth were cultured on to EMB agar, and characteristics *E. coli* suspected colonies were subcultured onto MacConkey agar to obtain pure bacterial colony ([Zadik et al., 1993](#)). Isolation of *E. coli* from beef samples was performed as per the method described in [BAM \(1998\)](#). A quantity of 10 gm of the beef sample was minced with the help of sterile scissors and mixed in 90 mL normal saline solution (pH 7.2) in screw cap bottle. Ten-fold serial dilutions were made up to 10⁻⁵ dilution in normal saline solution. A quantity of 0.1 mL inoculum from 10⁻³ and 10⁻⁴ dilutions have been cultured by spread plate technique on Eosin Methylene Blue agar (EMB) (Himedia, India) following incubation at 37°C for 24 h. Typical characteristics colony of *E. coli* on EMB agar as greenish metallic sheen was enumerated and isolated. Isolated organisms with supporting growth

Table 1. Oligonucleotide primers used in this study

Primers	Sequence (5'-3')	Target gene	Target size (bp)	References
ECO-1	GACCTCGGTTTAGTTCACAGA	<i>16S rRNA</i>	585	Schippa et al. (2010)
ECO-2	CACACGCTGACGCTGACCA			
STX1-F	CACAATCAGGCGTCGCCAGCGCACTTGCT	<i>stx1</i>	606	Talukdar et al. (2013)
STX1-R	TGTTGCAGGGATCAGTCGTACGGGGATGC			
STX2-F	CCACATCGGTGTCTGTTATTAACCACACC	<i>stx2</i>	372	
STX2-R	GCAGAACTGCTCTGGATGCATCTCTTGGTC			

characteristics of *E. coli* were subjected to sugar (dextrose, fructose, maltose, lactose, and sucrose) fermentation, MR-VP and indole production test following the procedure mentioned by [Cheesbrough \(1985\)](#).

DNA extraction: Bacterial DNA has been isolated using the method described by [Queipo-Ortun et al. \(2008\)](#). One mL of broth culture was centrifuged at 10000 rpm for 3 min; pellets were collected and mixed in 500 µL water by pipetting, boiled for 10 min followed by centrifugation at 10,000 rpm for 3 min. The supernatant was collected and used as template in PCR.

PCR to detect *16S rRNA*, *stx1*, and *stx2* gene: The isolated organisms that were cultural, morphologically and biochemically identified as *E. coli* were confirmed by PCR using genus-specific primers to detect *E. coli 16S rRNA* gene (**Table-1**). PCR was performed based on the scheme by [Schippa et al. \(2010\)](#). Briefly, 25 µL reactions contained 12.5 µL of 2X PCR master mix (Promega, USA), 1 µL of each primer (10 pmol/µL), 2 µL of template DNA and 8.5 µL of nuclease-free water. PCR amplification was performed on a thermocycler (Eppendorf Personal, Germany) with an initial incubation at 95°C for 3 min, a 30-cycle amplification of 94°C for 45 seconds, 58°C for 45 Sec and 72°C for 60 Sec and a final extension of 72°C for 3 min. The thermal condition to detect STEC gene was an initial denaturation of 94°C for 5 min, a 30-cycle amplification of 94°C for 1 min, 58°C for 1 min (59°C for *stx2*) and 72°C for 2 min and a final extension of 72°C for 5 min. The amplified products were resolved in 1.5% agarose gel, electrophoresed at 60 volts for 1 h ([Sambrook and Russel, 2001](#)) and examined under UV transilluminator using the 100-bp DNA ladder (Promega, USA) after stained with ethidium bromide.

Antibiotic sensitivity test: Antibiotic sensitivity test of isolated *E. coli* has been performed with standardized commercial antibiotic discs (Oxoid, UK) following Disc Diffusion Method ([Bauer et al., 1966](#)). Sensitivity to antibiotics were studied on Muller Hinton agar plates (Himedia, India) with ciprofloxacin (CIP) 5 µg, Amoxicillin (AMX) 30 µg, ampicillin (AM) 10

µg, tetracycline (Te) 30 µg, gentamicin (GM) 10 µg, nalidixic acid (NA) 30 µg, chloramphenicol (C) 30 µg and ceftriaxone (CRO) 30 µg. An amount of 0.1 mL freshly grown pure culture of *E. coli* in nutrient broth (Turbidity was compared with 0.5 McFarland standards) was poured on agar plates and allowed to spread gently over the entire surface with a glass rod spreader. After 5 min, the discs were placed at a distance of about 1 cm apart and incubated at 37°C for overnight. Based on the diameter of zones of inhibition produced around the antibiotic discs the inhibitory effect of the antibiotic on the growth of the culture was documented and evaluated according to [CLSI \(2015\)](#).

Statistical analysis: The correlation among the pathogens in fecal samples and beef samples were analyzed by Pearson correlation coefficient method using the R version 3.4.4

RESULTS

Detection of *E. coli*: In the present study, 42% (n=21/50) rectal swab and 32% (n=16/50) beef samples harbored *E. coli*. All the isolates upon overnight incubation at 37°C produced greenish black colored colonies with a characteristic metallic sheen on EMB agar, large bright pink colored colonies with lactose fermentation on MacConkey agar. *E. coli* suspected isolates were confirmed by detecting 16S rRNA gene amplified at 585-bp in PCR (**Figure 1**) using the primers ECO-1 and ECO-2 ([Hassan et al., 2014](#)). Presence of *E. coli* in beef is positively correlated with the presence of *E. coli* in rectal swab (R=0.8402) in each of the five slaughterhouses and the result is statistically significant at $P \leq 0.1$ (P-value is 0.074818) in Pearson's Correlation Coefficient (**Table 2; Figure 1**).

Detection of STEC: Among the sample tested, 14% (n=7/50) rectal swab and 6% (n=3/50) beef samples having Shiga-toxin producing *E. coli* respectively. The *stx1* gene was amplified in three samples (**Figure 2**) and *stx2* gene was in seven samples (**Figure 3**) at 606-bp and 372-bp, respectively ([Talukdar et al., 2013](#)).

Table 2. Detailed result of the experiment

Study area	Rectal swab		Beef samples			Value of R ² (Pearson's Correlation Coefficient)	P-value (at P≤0.1)	
	Samples	<i>E. coli</i>	STEC	Samples	<i>E. coli</i>			STEC
SH-1	10	4	2 (stx1)	10	3	-	0.7059	0.074818
SH-2	10	5	1 (stx2)	10	3	1 (stx2)		
SH-3	10	3	1 (stx1)	10	2	-		
SH-4	10	3	-	10	3	-		
SH-5	10	6	3 (stx2)	10	5	2 (stx2)		

SH=Slaughterhouse

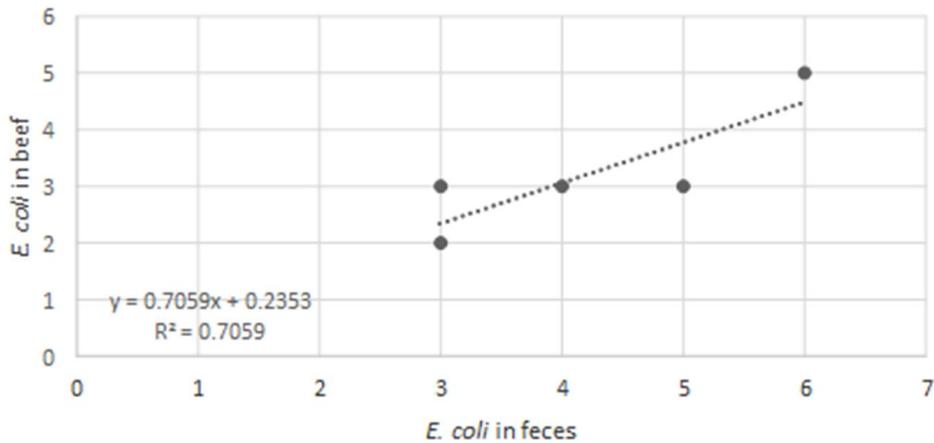


Figure 1. Pearson correlation coefficient showing the relationship among *E. coli* present in feces and meat of same slaughterhouse. The value of R², the coefficient of determination, is 0.7059. This is a strong positive correlation, which means that high X variable scores go with high Y variable scores (and vice versa)

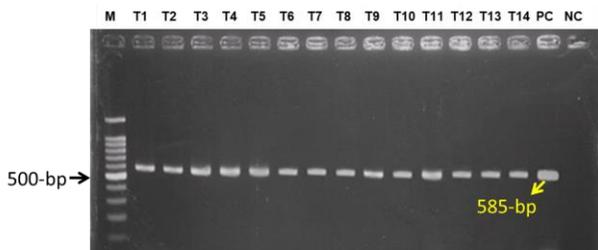


Figure 2. Electrophoretic analysis of gene *16S rRNA* in *E. coli*. Lane M: Molecular weight marker- 100 bp (Promega, USA); Lane T1-T14: Tested samples of *E. coli*, PC: Positive control; NC: Negative control.

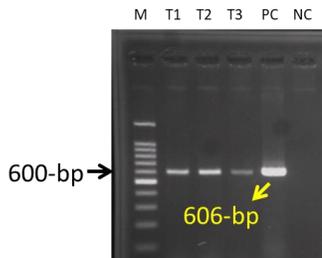


Figure 3. Electrophoretic analysis of gene *stx1*. Lane M: Molecular weight marker-100 bp (Promega, USA); Lane T1-T3: Tested samples of *E. coli*, PC: Positive control; NC: Negative control.

Table 3. Summary of STEC isolates and antibiotic sensitivity test.

ID	Source	Detected gene	Antibiotic resistance status
STEC-1	Bovine feces	<i>stx2</i>	AM, C, GM
STEC-2	Beef	<i>stx2</i>	AM, AMX
STEC-3	Bovine feces	<i>stx2</i>	AMX, GM
STEC-4	Bovine feces	<i>stx2</i>	CIP, GM, AMX
STEC-5	Bovine feces	<i>stx1</i>	C, Te
STEC-6	Bovine feces	<i>stx1</i>	GM
STEC-7	Bovine feces	<i>stx2</i>	AM, AMX, NA
STEC-8	Bovine feces	<i>stx1</i>	C, Te, GM
STEC-9	Beef	<i>stx2</i>	Te, GM, NA
STEC-10	Beef	<i>stx2</i>	AM, GM, NA

Antibiotic sensitivity test: Antibiotic sensitivity test against ten commonly used antibiotics in the country revealed that most of the isolated STEC strains were multidrug resistant against one or more antibiotics. The strains having *stx2* gene exhibited more drug resistance pattern than the strains harbored *stx1* gene (Table 2).

DISCUSSION

E. coli is the most common foodborne bacterial pathogen in humans and has been isolated from cattle at all stages

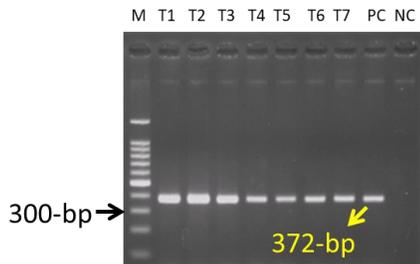


Figure 4. Electrophoretic analysis of gene *stx2*. Lane M: Molecular weight marker – 100 bp (Promega, USA); Lane T1-T7: Tested samples of *E. coli*; PC: Positive control; NC: Negative control.

of production. The shedding of the organisms is intermittent and can be challenging to detect, although they appear to be extended throughout the bovine population (Abd El-Atty and Meshref, 2007). This study revealed 42% beef cattle in slaughterhouses are the natural reservoir of *E. coli*. The prevalence obtained in this study is lower than our previous report in a dairy farm (Hassan et al., 2014), where the prevalence of *E. coli* was 75% in apparently healthy cattle, however, Ogunleye et al. (2013) reported 80% *E. coli* from the apparently healthy cattle in Nigeria. According to Masud et al. (2012), the prevalence of *E. coli* in the rectal swab of apparently healthy cattle between 2-3 years of age was 23.21% in Bangladesh. The prevalence varies according to geographical location and types of bovine animals sampled in the study. Detection of *stx* genes in *E. coli* suggests that the strains are potentially pathogenic (Arthur et al., 2002).

stx1 gene was detected in three isolates and *stx2* in seven isolates tested by PCR. In a routine diagnosis, it should be considered that a large number of samples might be processed in a relatively short period using the PCR assay (Cortez et al., 2006). We reported 43% STEC in the fecal specimen of dairy cattle in Bangladesh (Hassan et al., 2017), while beef cattle have lower prevalence (14%) of STEC in the present study. Pradel et al. (2001) isolated STEC from cattle, food products and HUS patients in France and reported that the strains were clearly different tested in both PFGE and the combination of *stx2*-RFLP, *stx2* variant, and plasmid profile analyses. Adwan and Adwan (2004) isolated 2.2% multidrug-resistant STEC from beef in Nigeria.

In Vietnam, 23% STEC was reported from cattle feces (Vu-Khac et al., 2008), whereas in India it was 2% in beef cattle and 7.26% in the calf (Mannan et al., 2006). In addition, Schmidt et al. (1999), reported that STEC belonging to serotypes O26, O103, and O111 having

close and unique clonal relationship. The lower prevalence of *stx1* gene in compared to *stx2* may have been caused by instability of the phages carrying *stx1* genes. Loss of *stx* genes in serial cultures is seen after long storage/culturing of the organism (Karch et al., 1999). At present, there are no data available on the outbreak of HUS and therefore the burden of STEC-associated HUS in Bangladesh is not elucidated yet. In order to evaluate the total burden of HUS in Bangladesh, it is recommended to perform a prospective study comprising all tertiary-level hospitals and some randomly selected secondary-level hospitals. All HUS cases admitted to these hospitals should be counted in the study. Stool samples should be tested for the existence of *stx* genes and STEC organisms together with serogroup O157.

Antimicrobial resistance has clearly inexorable alarming levels in human pathogens, but some fears have also been elevated concerning pathogens and commensals from animals (Cary et al., 2003). Multiple drug resistance was detected in all the STEC tested in this study. The worldwide overuse or misuse of antimicrobials in diverse fields, comprising human medicine and veterinary medicine as prophylactic supplements or growth-promoting agents in the feed of food animals, has produced antimicrobial resistance among bacterial pathogens and endogenous microflora (WHO, 2000). The purpose of antimicrobial susceptibility of clinical isolates and surveillance for resistant pathogens is often vital for the optimal antimicrobial therapy of infected patients. This need is becoming a more burning issue with increasing resistance and the emergence of multidrug-resistant microorganisms (Fluit et al., 2000). The findings of the present study ascertain that these organisms have been developed resistance for routinely prescribed antimicrobial drugs and pose considerable health hazards to the consumers unless prudent control measures are established. The isolation of this pathogen recommends that contaminated meat and meat products are sold to consumers and thus exposing them to foodborne hazards. Furthermore, it indicates that food hygiene and handling practices endure a great challenge in a developing country like Bangladesh.

CONCLUSION

The association between fecal prevalence of *E. coli* and carcass contamination indicates a role for control of *E. coli* in cattle on the farm to reduce and control the risk of human infection from ingestion of undercooked beef or cross-contamination of other foods of animal origin.

However, such a control program would also reduce the environmental contamination of *E. coli*, another potential source of human infection. Unfortunately, farmers in Bangladesh currently follow no effective measure to control pathogens in cattle. The finding in this study is the indication of the poor sanitary environment under which animals are reared, transported, slaughtered, processed and sold. The report of the presence of STEC in beef should make the conscious among relevant authorities that most foodborne illnesses may be due to unhygienic condition and mishandling of foods, while animal-borne pathogens introduced into the environment, lead to human illness associated with consumption of contaminated meat. Therefore, control strategies of foodborne pathogen and environmental pollution issues should be major targets in efforts to improve meat and food safety in developing, least developed and underdeveloped countries.

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CONFLICT OF INTEREST

There is no conflict of interest to declare.

AUTHORS' CONTRIBUTION

MSP, MM and MMM planned the study and conducted the actual research works. JH analyzed and interpreted the data. MSP and JH drafted the manuscript. MR and MTR helped in preparing, drafting and correcting of this manuscript. MBR and KHMNH critically checked the manuscript before publication. All the author read and approved before final submission.

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