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Original Article

Molecular based prevalence of shigatoxigenic *Escherichia coli* in rectal swab of apparently healthy cattle in Mymensingh district, Bangladesh

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ABSTRACT

Objective: Cattle are regarded as the principle reservoir of O157 and non-O157 shigatoxigenic *Escherichia coli* (STEC). Spreading of the STEC to human is primarily happens through contaminated meat, milk and their byproducts. The present study was aimed to explore the occurrence of STEC in the rectal swab of apparently healthy cattle.
Materials and methods: A total of 60 *E. coli* isolates that were previously isolated from the rectal swab of cattle were used in this study. DNA were extracted from the isolates and screened by PCR to detect *E. coli stx* (*stx1*, *stx2*), *ehx*A and *rfb*O157 genes. Representative amplicons of the PCR products were sequenced. The prevalence of the STEC was determined based on the detection of STEC specific *stx* genes. The prevalence data were further analyzed by SPSS to elucidate any difference among different demographic groups of the study population.
Results: Overall, 43.33% (n=26/60) of the isolates were found carrying *stx* genes. Based on the presence of *stx* and *ehx*A genes, 6 different types of STEC were identified, of which 20% (n=12/26) were carrying both *stx1* and *stx2* genes. None of the isolates was positive for *rfb*O157. The PCR amplicons were sequenced, and the nucleotide sequences were deposited in GenBank (accession: KM596779-KM596784).
Conclusion: In this study, non-O157 STEC were found highly prevalent in the local cattle. This study suggests that the apparently healthy cattle may act as a potential source of STEC infection for humans.

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KEYWORDS

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INTRODUCTION

Shiga toxigenic *Escherichia coli* (STEC), has become an increasing public health concern since its first identification in 1982 (Mainil and Daube, 2005). STEC becoming major concern for their association with hemolytic uremic syndrome and hemorrhagic colitis In human. Along with O157:H7 STEC infection, outbreaks and isolation of no-O157:H7 are increasing from different sources with time, and from 1983 to 2002 STEC infection with non-O157:H7 was recorded as approximately 70% (Brooks et al., 2005).

Stx is the major virulence property of STEC resulting host cell death by inhibiting protein synthesis. STEC produces one or more heterogeneous and immuno-logically non cross reactive Stxs (*stx1*, *stx2* or variants). Though *stx1* is identical to shigatoxin of *Shigella dysenteriae*, *stx2* shares only ~56% identity with *stx1* (Islam et al., 2008). In addition, some potential virulence genes *viz*., *ehx*A, *kat*P, *esp*P and and type II secreting system (*etp*D) has been reported in a ~90kb plasmid present in certain STEC strains (Farooq et al., 2009). Vast majority of enterohaemorrhagic *E. coli* (EHEC) associated with HUS harbors EHEC-*Hly*, a cytolysin, belongs to the RTX family (Schmidt et al., 1996; Bielaszewska et al., 2007). EHEC-Hly has the ability to injure microvascular endothelial cells (Aldick et al., 2007).

STEC (O157 and non-O157) has been reported in the intestinal tract and dropping of different animal and birds including its major reservoir as the cattle and sheep (Griffin and Tauxe, 1991; Hazarika et al., 2007; Jomezaden et al., 2009). Although ruminants harbor STEC in their intestine, they are not affected by shigatoxins, due to the lack of specific receptors for shigatoxins on their cell surface. There are reports suggesting that ruminants could shed and spread STEC to humans through fecal contamination of meat and milk (Elder et al., 2000; Asakura et al., 2001; Naidu et al., 2007). Additionally, person to person contact is also well documented as a mode of transmission of STEC (Rodolpho and Marin, 2007).

Prevalence of STEC in various sources have been reported worldwide including Bangladesh (Chapman et al., 1994; Fratamico et al., 2004; Cookson et al., 2006; Islam et al., 2007, 2008; Kesava et al., 2011; Islam et al., 2014; Hamza et al., 2017). However, no work on the prevalence of STEC in the rectal swab of apparently healthy cattle has yet been reported in Mymensingh, Bangladesh. The present study is describing the prevalence of non-O157 STEC in the rectal swab of apparently healthy cattle based on PCR and sequencing.

MATERIALS AND METHODS

Ethical statement: Not applicable.

Bacterial strains and cultural conditions: Previousl we isolated *E. coli* from rectal swab of apparenty healthy cattle in Mymensingh (Hassan et al., 2014). Sixty *E. coli* strains (n=60) from that previous study were selected randomly and used in this study for detection of STEC.

DNA extraction and detection of virulence genes: DNA from the pure isolates was extracted by boiling (Hassan et al., 2014). The presence of virulent genes (*e.g.*, *stx*1*, stx2*, *ehx*A and *rfb*O157) was detected using specific primers listed in **Table 1**. PCR reaction mixtures were adjusted to 25 µL with PCR master mix (Promega, USA) and 10 pmol of each primer. PCR was performed with an initial denaturation at 94°C for 5 min, followed by 30 cycles of amplification [denaturation: 94°C for 1 min; annealing: 1 min at varying temperature depending on the target genes (*i.e.*, 58°C for *E. coli* 16S rRNA gene, 61°C for *stx1*, 59°C for *stx2*, 49°C for *ehx*A, and 48°C for *rfb*O157); extension: 72°C for 2 min] with a final extension for 5 min at 72°C. PCR products were separated in 2.0% agarose and DNA was visualized in UVsolo TS Imaging System (Biometra, Germany).

Table 1. Oligonucleotide primers used in this study

Target gene	Primer name	Sequence (5' - 3')	Product size (bp)	References
stx1	stx1F	CACAATCAGGCGTCGCCAGCGCACTTGCT	606	Talukdar et al. (2013)
	stx1R	TGTTGCAGGGATCAGTGGTACGGGGATGC		
stx2	stx2F	CCACATCGGTGTCTGTTATTAACCACACC	372	
	stx2R	GCAGAACTGCTCTGGATGCATCTCTGGTC		
*ehx*A	hly-EHECAF	GAGCGAGCTAAGCAGCTTG	889	Wieler et al. (1996)
	hly-EHECAR	CCTGCTCCAGAATAAACCACA		
*rfb*O157	rfbO157F	AAGATTGCGCTGAAGCCTTTG	497	Sánchez et al. (2010)
	rfbO157R	CATTGGCATCGTGTGGACAG		

Sequencing of the PCR amplicons: Randomly selected PCR products of *stx1, stx2*, and *ehx*A were subjected to commercial sequencing from 1st BASE Laboratories-SdnBhd, Malaysia. The qualities of the obtained sequence were checked and processed using Chromas 2.23 and SeqMan II (DNASTAR). BLAST search was carried out to determine the identity of the nucleotide sequences using the NCBI, BLAST server. The sequences were deposited to GenBank.

Data analysis: Data collected during sampling were entered and statistically analyzed using SPSS version 17 (Chicago: SPSS Inc.). We applied *Chi*-square (χ^2) test to find out the significant relationship between two interrelated qualitative variables. Fisher's exact test was used for the contingency table whose cell frequency was less than 5. The associations between interrelated variables were measured by calculating the contingency coefficient and odds ratio (OR). *P* values ≤0.05 was considered as significant.

RESULTS

Prevalence of STEC and their virulence genes

The prevalence of the STEC was determined based on the PCR amplification of *stx* (*stx1, stx2*) genes. Among the 60 isolates, 43.33% (n=26/60) were found to be positive for *stx* genes (**Figure 1-2**). The primers targeting hemolysin *i.e., ehx*A and *E. coli* O157 specific O antigen *i.e., rfb*O157 were also used. Among the 60 isolates, 10% (n=6/60) were found to be positive for *ehx*A (**Figure 3**). None of the isolates was positive to *rfb*O157. Prevalence analysis based on the demographic factors revealed higher prevalence of non-O157 STEC in local cattle aged above 3 years that were maintained under unorganized farming (management) systems (**Table 2**).

*Distribution of stx1, stx2 and ehx***A** *among the STEC*

Among the 60 isolates screened, STEC harboring 6 different combinations of target genes were identified among which isolates bearing both *stx1* and *stx2* are predominant (**Table 3**). The PCR amplicons of the target genes were sequenced. Upon alignment, all the *stx*1 gene sequences were found to be identical and 3 *stx2* and 2 *ehx*A gene sequences were found dissimilar at different level. One (1) *stx1* gene sequence, 3 of the *stx2* gene sequences and 2 of the *ehx*A gene sequences were deposited to GenBank (accession: KM596779, KM596780, KM596781, KM596782, KM596783, KM596784).

DISCUSSION

The prevalence of non-O157 STEC in the rectal swab of apparently healthy cattle was found to be 43.33% as revealed by PCR based approach. Comparing the other studies performed in Bangladesh or abroad (Islam et al., 2008; Cookson et al., 2006; Menrath et al., 2010; Islam et al., 2014) the prevalence of STEC obtained in the present study is higher. This variation might be related with

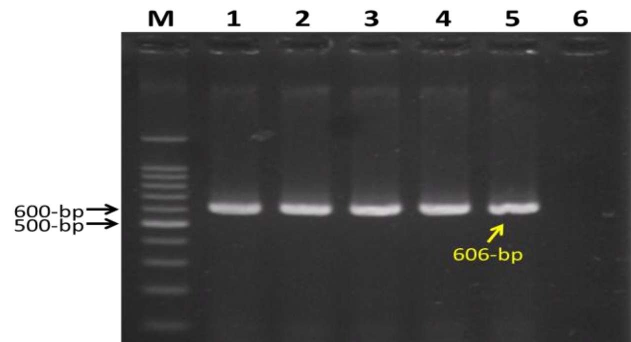

Figure 1. Representative figure showing the amplification of *stx1* gene using the primers_stx1F and stx1R. M=100 bp DNA ladder, 1-4 = Test samples, 5 = Positive control, 6 = Negative control.

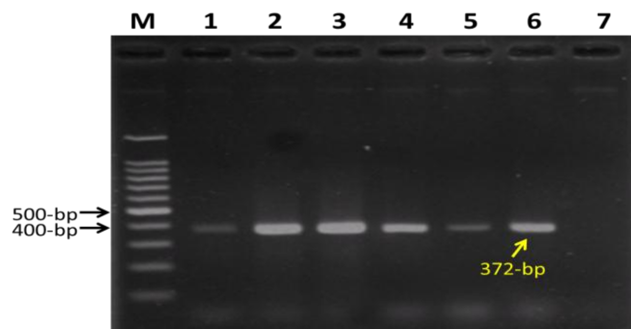

Figure 2. Representative figure showing the amplification of *stx*2 gene using the primers stx2F and stx2R. M=100 bp DNA ladder, 1-5=Test samples, 6=Positive control, 7=Negative control.

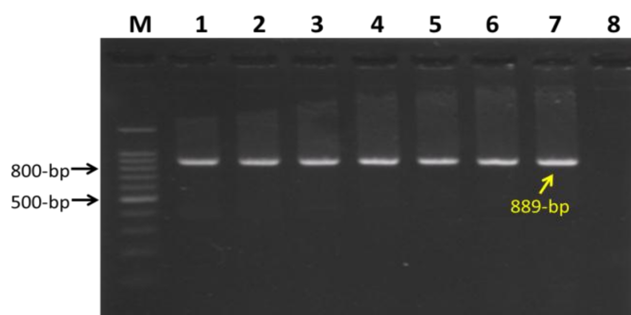

Figure 3. Representative figure showing the amplification of *ehx*A gene using the primers hlyEHECF and hlyEHECR. M=100 bp DNA ladder, 1-6=Test samples, 7=Positive control, 8=Negative control.

Table 2. Prevalence of STEC in the rectal swab of cattle

Parameters		Positive	Negative	Total (n= 60)	*P* value (χ2-test)*	Contingency coefficient (CC) value
Age	0-3 yrs	9	22	31	0.021	0.286
	3-above	17	12	29		
Odds Ratio (0-3/above 3): 0.289 (CI: 0.099-0.843)						
Sex	Male	7	19	26	0.025	0.278
	Female	19	15	34		
Odds Ratio (Male/Female): 0.291(CI: 0.097-0.873)						
Breed	Local	7	2	9	0.032	0.280
	Cross	19	32	51		
Odds Ratio (Local/Cross): 5.895 (CI: 1.109-31.340)						
Management systems	Organized	11	29	40	0.000001	0.412
	Unorganized	15	5	20		
Odds Ratio (organized/Unorganized): 0.126 (CI: 0.037-0.431)						

**Data were analyzed based on the age, sex, breed and management systems of the animals. P values were calculated using Pearson's Chi-Square test except the value in case of Breed where Chi-square test was applied using Fisher's Exact Test. P values below 0.05 (P≤0.05) was considered as significant.*

Table 3. Distribution of *stx1*, *stx2* and *ehx*A among the STEC (N=60)

Target gene	No. of samples positive (%)
stx1	2 (3.33)
stx2	6 (10.00)
stx1 + *stx2*	12 (20.00)
stx1 + *ehx*A	1 (1.67)
stx2 + *ehx*A	2 (3.34)
stx1 + *stx2* + *ehx*A	3 (5.00)
Total:	26 (43.33)

several factors such as the sample size and demographic characteristics of the study population. The prevalence of O157 *E. coli* as revealed in this study is inclined with the previous report of Hussein and Sakuma (2005).

In the present study, we found STEC positive for either *stx1* or *stx2* singly, while some were both *stx1* and *stx2* positive. In addition, *ehx*A was also identified in some of these *stx1* and/or *stx2* positive SETC. However, it was not surprising to observe these kind of findings since occurrence of either *stx1* or *stx2* or both in a single strain of STEC has earlier been identified from cattle (Renter et al., 2007).

This study also revealed 6 sub-groups of STEC based on the presence of different genes screened, where the prevalence of *stx1* or *stx2* alone was lower than the finding of Kesava et al. (2011). The prevalence of *stx1* alone was lower but occurrence of *stx2* alone or in combination with *stx1* was higher than that of Cookson et al. (2006). This variation might be due to differences in study population and location.

Prevalence of non-O157 STEC was found significantly higher in local cattle over 3 years of age maintained under unorganized farming system compared to organized farm which might be resulted from recurrent exposure of the animals to STEC contaminated feed materials. However, it's difficult to make certain inference on the potential risk group of cattle to STEC based on this small number of samples analyzed in this study. Further studies covering more areas need to be focused to identify the potential risk group of animals and minimize the spread of STEC to human being.

CONCLUSION

Cattle are regarded as the major reservoir of STEC for human infection. About 43.33% of the rectal swab collected from the apparently healthy cattle was found to be positive for STEC in Mymensingh, Bangladesh. All the STEC isolates revealed in this study belongs to non-O157. In addition to the presence of *stx* encoding genes, hemolysin encoding genes have also been detected in thee STEC isolates. The occurrence of STEC in the rectal swab of apparently healthy cattle signifies that these cattle could be the potential source for pathogenic *E. coli* to human.

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CONFLICT OF INTEREST

The authors declared that they had no conflict of interests.

AUTHORS' CONTRIBUTION

JH, MSP and TK conducted main experiments. JH prepared the first draft of this manuscript. KHMNHN and MTR corrected and approved the manuscript for publication. All the authors read and approved the final version of the manuscript.

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