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*Original Article*

# Prevalence of shiga toxin-producing Escherichia coli in buffaloes on smallholdings in coastal area in Bangladesh

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## *Abstract*

Shiga toxin-producing *Escherichia coli* (STEC) is an important causal agent of food borne diseases. Although the presence of STEC has been reported in cattle, goat, and sheep in previous studies in Bangladesh, there is seemingly no information on sorbitol non-fermenting STEC (SN-F STEC) carriage in buffalo. We aimed to assess the prevalence of SN-F STEC and to identify common virulence genes in SN-F STEC strains from apparently healthy buffaloes on smallholdings in coastal area in Bangladesh. Total 308 buffaloes on smallholdings in Patenga and Anwara Upazila, Chattogram division, Bangladesh were randomly sampled by collecting a swab from the recto-anal junction of each animal. SN-F *E. coli* was identified based on colony morphology (colorless) yielded on Cefixime tellurite sorbitol MacConkey (CT-SMAC) agar. SN-F *E. coli* was detected in 33 [10.7%, 95% Confidence Interval (CI): 7.7-14.7)] of the animals sampled. Among them, 12 (3.9%, 95% CI: 2.2-6.8) were STEC positive. These isolates were characterized for the presence of *stx* (*stx1* and *stx2*) and common virulence genes, *eae* and *hlyA* by the Polymerase Chain Reaction (PCR). Fourteen isolates exposed only one gene, five isolates harbored two genes and two isolates carried three genes. Healthy buffaloes carry STEC, though its prevalence is comparatively lower than from cattle could pose a risk to public health reared on smallholdings.

**Keywords:** Buffalo, *Escherichia coli*, *rfb* O157, shiga toxin, virulence genes

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## Introduction

*Escherichia coli* is a normal commensal bacteria inhabit the gut of animals and humans. However, some strains of *E. coli* are responsible for intestinal and extraintestinal infections (Yassin *et al*., 2017). There are six major groups of *E. coli* based on the pathogenicity such as enterohemorrhagic *E. coli* (EHEC) which harbors *stx* genes and producing shiga toxin, recognized as shiga toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Nataro & Kaper, 1998). STEC, one of the foremost foodborne zoonotic pathogens worldwide (Beutin *et al*., 2004; Hyochin & Arun, 2008). The virulence of STEC depends on the products of 4 genes: *stx1* and *stx2* (for the production of shiga toxin 1 and 2, respectively), *eaeA* (for the production of adhesion/intimin) and *hlyA* (for the production of enterohemolysin) (Karch *et al*., 1998).

STEC is one of the pathogenic groups having zoonotic significance because it causes severe clinical diseases in humans, unlike its reservoirs, such as cattle, where it lives and multiplies without causing any harm. STEC's potential to cause human disease is related to the development of Shiga toxins, which prevent host cells from producing protein, resulting in cell death (Karmali *et al*. 2010). STEC O157:H7, the most reported serotype that can cause diseases ranging from mild diarrhea to hemolytic uremic syndrome (HUS), kidney failure and death in human (Mead & Griffin, 1998). STEC O157:H7 received worldwide attention because of their association with numerous outbreaks in human, several other serogroups of STEC (O26, O91, O103, O104, O111, O113, O121, O123 and O145) have also been isolated from severe outbreaks of human disease in different countries (Hussein 2007; Espie *et al*. 2008; King *et al*. 2009). Food-borne zoonoses like STEC infections are transmitted via contaminated food, like minced meat, sausage, hamburger, raw and unpasteurized milk and dairy products (Normanno, 2007).

Cattle are well known natural STEC reservoirs. Though, other domestic ruminants like sheep, goats and buffalo are also important (Hawley, 2016) and play a vital role in the epidemiology of human infections (Karmali *et al*., 2010). As STEC reservoir, buffaloes have been identified in many countries in Asia, Europe, and America (Amézquita-López *et al*., 2018; Oliveira *et al*., 2007; Persad & Lejeune, 2014; Srivani *et al*., 2017). STEC had already been discovered in healthy cattle, buffaloes, and goats on smallholdings in Bangladesh (Islam *et al*., 2015; Mannan *et al*., 2020; Gupta *et al*., 2016), despite 38% buffalo sampled after the slaughter in an urban area of Bangladesh found STEC positive (Islam *et al*., 2008). However, published information on the prevalence of STEC in buffaloes reared in smallholdings seems scanty, if not absent. Here, we describe the SN-F STEC in buffaloes reared under smallholdings in Patenga and Anwara Upazila, Chattogram, Bangladesh.

## Materials and Methods

***Ethical statement:*** Considering animal welfare, sample collections was approved by the Institutional Animal Ethics Committee, Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh (Ethical approval no: 0213/06:2013-2014).

***Study area and duration:*** A cross-sectional survey was conducted in the coastal area of Patenga and Anwara Upazila, Chattogram District, one of the sixty four districts in Bangladesh under Chattogram Division, Bangladesh from June 2017 through July 2016.

***Sample size calculation:*** The minimum sample size, estimated primarily was 100 based on the formula $\pi (1-\pi)/e^2$, where $\pi$ is the prevalence and e is the standard error (Kirkwood & Sterne, 2003). As on the prevalence, there is no published information of SN-F STEC in buffaloes reared on smallholdings, we considered the prevalence 0.5 with standard error 0.05. However, finding a low prevalence in the initial phase of screening of the samples, we decided to sample around 300 animals, and in the end, we sampled 308 buffaloes by simple random sampling at one animal each from 308 selected smallholdings (small farm operating under a small-scale agriculture model). The number of animals vary in each smallholdings.

***Sampling and sample processing****:* Total 308 fecal swab samples from recto-anal junction of apparently healthy buffaloes were collected. A sterile screw capped falcon tube having buffered peptone water (BPW; Oxoid, UK) was used to transfer the swab sample into an ice box immediately to the laboratory, Department of Microbiology and Veterinary Public Health, CVASU, Chattogram, Bangladesh.

***Bacteriological processes:*** A sample in BPW was incubated for primary enrichment at 37°C for 24 h. One loopful of positive enrichment was plated onto MacConkey agar (Oxoid, UK) and incubated overnight at 37°C. Large pink colored colonies yielded on MacConkey agar were streaked onto Eosin Methylene Blue (EMB) agar (Oxoid, UK), incubated at 37°C for 18-24 h. Only the distinctive metallic green colonies produced on EMB agar were subjected to a number of biochemical tests [catalase (+ve), oxidase (-ve), Indole-Methyl Red-Voges Proskauer-Citrate (+ + - -) and urease (-ve) to confirm the growth of *E. coli* (Wani *et al*., 2004). Five cross-sectional colonies of *E. coli* on an EMB agar were streaked onto a CT-SMAC agar (Oxoid, UK), selective medium for STEC O157, incubated at 37°C for 24 h. Any growths of colorless colonies indicated the presence of sorbitol non-fermenter (Thrusfield, 2005; Krishnan *et al*., 1987). Presumptively positive SN-F EC isolates obtained by seeing the colorless colonies were preserved in Luria-Bertani (LB) broth with 15% glycerin at −80°C.

***DNA extraction:*** For the PCR, DNA was extracted by boiling method (Sánchez *et al*., 2010) with slight modifications from the selected SN-F colonies. A loopful of *E. coli* colonies were picked up and mixed with 200 µl of deionized water into 1.5 ml sterile Eppendorf tube followed by boiling at 99°C for 15 min, centrifugation at 15000 rpm for 2 min. As a DNA

template, the collected supernatant was used and stored at −20°C.

***Detection of shiga toxin and other virulence genes:*** All the SN-F EC isolates were investigated by uniplex PCR through specific primers for *rfb*, *stx1*, *stx2*, *eae*, and *hlyA* genes described in previous studies (Table-1). The presence of the *rfb* gene was verified to assess whether or not the isolates belonged to serogroup O157. Each PCR reaction was accomplished with 25 µl reaction volume comprising 1 µl DNA template, forward and reverse primer (0.5 µl each), GoTaq master mix (Promega, USA) (12.5 µl) and deionized water (10.5 µl). PCR amplifications were performed by a Thermo-cycler (2720 thermal cycler, Applied Biosystems, USA). Previously published thermal conditions were used to detect the genes (Table-1). Products of PCR, electrophoresed by running in 1.5% agarose gel stained with ethidium bromide (Sigma-Aldrich, USA). As a positive control, an SN-F STEC strain preserved from a previous study was used (Islam *et al*., 2014).

**Table 1** Primers used to identify *rfb*, *stx1*, *stx2*, *eae* and *hlyA* genes in the isolates obtained

| Target gene | Primer sequences (5´-3´) | Annealing temp (°C) | Size of product (bp) | Reference |
|---|---|---|---|---|
| *rfb* | F: CGGACATCCATGTGATATGG<br>R: TTGCCTATGTACAGCTAATCC | 58 | 259 | Desmarchier *et al*., 1998 |
| *stx1* | F: ACACTGGATGATCTCAGTGG<br>R: CTGAATCCCCCTCCATTATG | 58 | 614 | DesRosiers *et al*., 2001 |
| *stx2* | F: CCATGACAACGGACAGCAGT<br>R: CCTGTCAACTGAGCAGCACTT | 58 | 779 | Manna *et al*., 2006 |
| *eae* | F: CCCGAATTCGGCACAAGCAT<br>R: CCCGGATCCGTCTCGCCAGTA | 59 | 881 | Oswald *et al*., 2000 |
| *hlyA* | F: ACGATGTGGTTTATTCTGGA<br>R: CTTCACGTGACCATACATAT | 58 | 165 | DesRosiers *et al*., 2001 |

***Statistical analysis:*** Data were maintained into a spreadsheet of Microsoft Excel 2010. Online Epitools epidemiological calculator (Sergeant, 2019) was used for the estimation of 95% confidence interval (CI).

## *Results*

***SN-F E. coli:*** A total of 77 (77/308, 25%) samples yielded bright pink colored colonies on MacConkey agar plate and on EMB agar plate produced metallic sheen colonies. When at least five homogenized cross-sectional colonies from EMB agar were streaked onto CT-SMAC agar, 33 (33/308, 10.7%) isolates produced pure SN-F (colorless) colonies.

***SN-F STEC:*** The presence of SN-F EC was detected in 10.7% (n=33, 95% CI: 7.7-14.7) of the samples.

All 33 SN-F EC isolates were screened by PCR assay for the presence of *rfb*, *stx1* and *stx2* genes. Only four isolates (1.3%, 95% CI: 0.4–3.4) were positive for the *rfb* gene, indicating that they could belong to serogroup O157. Of the total SN-F *E. coli* isolates, 8 and 4 were harbored to the *stx1* and *stx2* gene, respectively. None of them, however, contained both the genes. Therefore, the prevalence estimates for the SN-F EC containing the *stx1* gene and the *stx2* gene were 2.6% (n=8/308, 95% CI: 1.2–5.1) and 1.3% (n=4/308, 95% CI: 0.4%–3.4%), respectively. Thus, regardless of the distribution of both *stx1* and *stx2* genes in the isolates, the prevalence of buffalo as carriage for SN-F STEC was 3.9% (n=12/308, 95% CI: 2.2-6.8).

***Strains having stx/eae/hlyA gene:*** Of the 12 SN-F STEC isolates, whether they carried either the *stx1* or the *stx2* gene, 5 harbored the *eae* while 2 had the *hlyA* gene. One isolate carried out *stx2*, *eae* and *hlyA* genes. On the contrary, four and three SN-F non-STEC isolates were positive to the *eae* and *hlyA* gene, respectively (Table 2).

## *Discussion*

In our study, we have reported the prevalence of SN-F *E. coli* and SN-F STEC in buffaloes reared in smallholdings in Bangladesh, and the results revealed that approximately 10.7% and 3.9% population harbored them, respectively. Based on vigorous literature searches, authors claimed that it might be the first report from apparently healthy buffaloes on SN-F STEC in Bangladesh. In a preceding study from the same region, Islam *et al*. (2015) reported that cattle on smallholdings were positive for SN-F *E. coli* (8 5%) and SNF STEC (5 4%). Islam *et al*. (2008) reported 20.1% prevalence of STEC in cattle from the same area. In other countries, Mahanti *et al*. (2013), Mohammad *et al*. (1985), Oliveira *et al*. (2007) and Vu-Khac & Cornick (2008) reported higher prevalence of STEC in buffaloes. We used CT-SMAC to isolate all bacteria from the STEC O157 serogroup as well as other SN-F types (Griffin, 1991). The presumptively positive isolates were confirmed by PCR assay for the presence of *stx* and other virulent genes. This disparity might be due to the difference in bacterial culture, uses of molecular testing, geographical location, immune status and management practices of the animals.

In this study, compared with *stx2*, the number of *stx1* producing SN-F STEC isolates was 2 times higher (Table 2). The finding of this study is in concurrence with the earlier report in Bangladesh by Islam *et al*. (2008), the *stx1* gene was reported in a large number (8.6%) than *stx2* gene (6.9%) in buffaloes. To the contrary, Mahanti *et al*. (2013), Oliveira *et al*. (2007) and Vu-Khac & Cornick (2008) stated a higher prevalence of the *stx2* gene from buffalo compared with the *stx1* gene in some other countries. We did not look at risk variables in any way for the differences of *stx1* and *stx2*. Additional epidemiological research is needed to determine the risk variables and their relationship to

STEC carriage in animals. However, these variations might be attributed to different factors such as age of buffaloes, diet, immune response, environmental conditions and overall farm management.

**Table 2** Dissemination of the *stx1* and *stx2*, and two other virulent genes, *eae* and *hlyA*, in the 33 SN-F EC isolates

| Isolate ID | *stx1* | *stx2* | *eae* | *hly* | *rfb* |
|---|---|---|---|---|---|
| 13 | - | - | - | - | + |
| 16 | - | + | + | - | + |
| 20 | - | - | + | - | - |
| 24 | + | - | - | - | - |
| 26 | - | - | - | - | - |
| 31 | - | - | - | - | - |
| 33 | - | - | - | - | - |
| 41 | - | - | - | - | - |
| 46 | - | - | + | - | - |
| 50 | - | - | - | - | - |
| 71 | - | - | + | - | - |
| 74 | - | - | - | + | - |
| 79 | - | - | + | - | + |
| 91 | + | - | + | - | - |
| 96 | - | - | - | - | - |
| 100 | - | + | + | + | - |
| 106 | - | - | - | + | - |
| 110 | + | - | + | - | - |
| 116 | + | - | + | - | - |
| 131 | + | - | - | - | - |
| 151 | - | - | - | - | - |
| 159 | + | - | - | - | - |
| 179 | - | - | - | - | + |
| 191 | - | - | - | - | - |
| 211 | + | - | - | - | - |
| 219 | - | - | - | - | - |
| 251 | - | + | - | - | - |
| 266 | - | - | - | + | - |
| 274 | - | - | - | - | - |
| 286 | - | + | - | + | - |
| 291 | - | - | - | - | - |
| 296 | - | - | - | - | - |
| 298 | + | - | - | - | - |

+, present; -, absent

Among the 12 SN-F STEC isolates the *eae* gene was carried by 5 (41.7%) and the *hlyA* by 2 (16.7%) isolates. The outer membrane protein, intimin (*eae*) is a virulence-associated factor that induces attaching and effacing lesions in the intestinal mucosa (Kaper, 1998). Enterohaemolysin, a pore-forming cytolysin encoded by the plasmid-borne gene *hlyA*, is another component that contributes to STEC pathogenicity (Schmidt, 1995). According to several investigators, the *eae* gene presence in the isolates carrying either *stx1* or *stx2* or both can make them more virulent in humans to cause severe clinical diseases (Beutin *et al*., 2004; Blanco *et al*., 2004). Therefore, a higher frequency of the gene *eae* in the STEC isolates from buffalo population in this study suggested that these isolates might pose a greater zoonotic risk to humans. The SN-F STEC isolates were seemingly diverse in this study due to having different combinations of the two *stx* producing genes and due to the occurrence of different combinations of two more virulence genes, *eae* and *hlyA*. However, the prevalence of STEC O157 seemed to be negligible as only 0.3% of the STEC and about 1% isolates from non-STEC, respectively, was positive for the serogroup O157, in agreement with a previous study in cattle population which also revealed a similar low proportion, 0.4% (Islam *et al*., 2015). O157 is a significant serotype that can cause colitis, HUS and sometimes death in humans.

In summary, 10.7% and 3.9% healthy buffaloes are reservoirs for SN-F *E. coli* and SN-F STEC in smallholdings, respectively. The occurrence of the *eae* and *hlyA* genes in the SN-F STEC suggests that these strains would be more virulent in humans and thus would pose a greater zoonotic risk. However, the prevalence of SN-F STEC belonging to serogroup O157 seems to be very low. Further studies are required to ascertain the sources and risk factors associated with human infections caused by SN-F STEC from buffaloes.

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