Occurrence of Shiga toxin-producing Escherichia coli carrying antimicrobial resistance genes in sheep on smallholdings in Bangladesh

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Abstract
Background: Shiga toxin-producing Escherichia coli (STEC) are zoonotic foodborne pathogens and a significant concern with the emergence of antibiotic resistance. Close human contact might have a higher chance of being transmitted to humans from sheep if the sheep population is a potential reservoir of antimicrobial-resistant STEC. Therefore, this study aimed to examine the sheep population in rural Bangladesh for antimicrobial-resistant STEC.

Methods: We screened 200 faecal samples collected from sheep in three Upazilas from the Chattogram district. Randomisation of sampling was not performed due to the smaller flock size (two to six animals per smallholding). Phenotypically positive E. coli isolates were examined for two Shiga toxin-producing genes – stx1 and stx2. PCR-positive STEC isolates were investigated for the presence of antimicrobial resistance (AMR) genes – blaTEM, sul1 and sul2.

Results: In total, 123 of the 200 tested samples were confirmed positive E. coli using culture-based methods. PCR results show 17 (13.8%) E. coli isolates harboured ≥ one virulent gene (stx1 or/and stx2) of STEC. The AMR profile of STEC isolates was determined utilising the disc diffusion method. Of the STEC isolates, 82, 76, 71 and 71% were susceptible to chloramphenicol, gentamicin, ciprofloxacin and ampicillin. In contrast, 47% of isolates were resistant to trimethoprim–sulfamethoxazole, and 41% were resistant to amoxicillin. In addition, six of the tested STEC isolates exhibited the blaTEM gene; eight STEC isolates had the sul1 gene, and the sul2 gene was detected in ten STEC isolates.

Conclusion: To our knowledge, this study is the first to reveal a substantial percentage of STEC isolated from sheep in rural Bangladesh harbouring AMR genes.

Key Words
Sheep, E. coli, antimicrobial resistance
1  |  INTRODUCTION

Antimicrobial resistance (AMR) in foodborne pathogens can be monitored by resistance in the key species of AMR, *Escherichia coli* (Cheney et al., 2015). *E. coli* is an asymptomatic intestinal inhabitant in food-producing animals. However, some *E. coli* variants can cause diarrhoeal disease and extra-intestinal infections by expressing several specific virulence factors known as pathogenic *E. coli* (Kaper et al., 2004; Nataro & Kaper, 1998; Pitout, 2012).

Shiga toxin-producing *E. coli* (STEC) is a pathogenic *E. coli* strain that can cause severe illness in humans, including life-threatening haemolytic uremic syndrome (Byrne et al., 2015). Approximately three-quarters of reported STEC infections in human is attributed to domestic ruminant (Mughini-Gras et al., 2018). Therefore, cattle and other ruminants such as sheep and goats are considered the most common reservoirs of STEC. This pathogen can be transmitted to human via direct contact with an infected animal, but foods (meat, milk and beverages or water) contaminated with faecal material is considered the main route of transmission (Fairbrother & Nadeau, 2006; Jones et al., 2019; Monaghan et al., 2012; Verhaegen et al., 2016).

Pathogenic strains of *E. coli* are becoming increasingly resistant by acquiring resistance genes derived from conjugative plasmids. Many of these resistance genes are found on mobile genetic elements that can be transmitted to other bacteria belonging to the same or different species, such as salmonella and campylobacter (EFSA, 2016; Allen et al., 2010). In recent years, widespread resistance to various antimicrobial in *E. coli* has been reported in humans and non-human sources (Nji et al., 2021; Rybak et al., 2022). Therefore, in addition to the recent focus on ‘One Health’ aspects of AMR, surveillance for antimicrobial-resistant *E. coli* in animals becomes more crucial to explore the extent of the public health risk posed by pathogens of zoonotic origin. Furthermore, monitoring the incidence and prevalence of AMR in STEC in healthy animals may measure the spread of resistant strains and resistance genes from animals to humans. Hence, crucial to revise and implement new control measures for antibiotic resistance.

Previous studies reported the prevalence of STEC in various livestock species in Bangladesh (Gupta et al., 2018; Hassan et al., 2017; Gupta et al., 2016; Islam et al., 2015). A high proportion of these STEC was resistant to sulfonamide drugs and beta-lactamase antibiotics (Gupta et al., 2013; Islam et al., 2013; Islam et al., 2015; Gupta et al., 2016; Gupta et al., 2017 and 2018). However, there is no report on the prevalence of STEC in sheep. In addition, there is no Bangladeshi study investigating antibiotic resistance at the gene level. Therefore, the current study aimed to determine the antibiotic resistance genes and explore STEC occurrence in faecal samples of healthy sheep in Bangladesh. *E. coli* isolates were screened for major virulence genes: *stx1* and *stx2*, and the presence of three AMR genes: *blaTEM*, *sul1* and *sul2* was determined.

2  |  MATERIAL AND METHODS

2.1  |  Sample collection

A total of 200 faecal samples were collected from 200 sheep from 100 smallholdings in three upazilas (an upazila is a subdistrict level area) of Chattogram district, Bangladesh, between June and December 2018. The number of sheep sampled from a smallholding varied between one and three depending on the flock size. The samples were collected from apparently healthy sheep of all ages, sexes and breeds on small-holder farms. For each animal, data on age, breed, sex, health status and previous history of antibiotic use were collected using a structured questionnaire by interviewing individual owner. Faecal materials from each animal were collected by inserting a sterile cotton swab into the recto-anal junction. After collection, swabs were dipped into buffered peptone water and stored in an icebox (4°C). Collected swabs were shipped immediately to Poultry Research and Training Center Laboratory at Chattogram Veterinary and Animal Sciences University. No ethical approval was obtained because this study did not contain patient data and only involved non-invasive procedures, that is, faecal samples. However, the animals were handled with care by experienced veterinary practitioners during sample collection. In addition, informed consent through verbal communication was taken from all farm owners before sample collection, assuring that sheep would not be harmed during sampling.

2.2  |  Culture and *E. coli* isolation

A presumptive *E. coli* isolate was identified based on the growths in selective media, as described previously (Gupta et al., 2017; Gupta et al., 2018). Briefly, a sample in buffered peptone water was incubated at 37°C for 24 h in an incubator for enrichment. Each enriched sample was streaked onto MacConkey agar (Oxoid), and the plate was incubated at 37°C for 24 h. Five well isolated bright pink colonies from MacConkey agar sub-cultured onto tryptic soy broth (TSB), incubated at 37°C for 6 h in a shaker incubator. Growths from TSB were again inoculated on the MacConkey agar. One well isolated pink coloured colony was then onto an Eosin Methylene Blue (EMB, Oxoid) agar plate, incubated at 37°C for 24 h to examine the characteristic metallic green sheen produced by *E. coli*. An isolate producing green colonies with a metallic sheen on EMB agar was confirmed as *E. coli*. All confirmed *E. coli* isolated were preserved at −80°C in LB broth with 15% glycerine until assessment for virulence genes.

2.3  |  Detection of Shiga toxin-producing genes

Total DNA was extracted from confirmed *E. coli* isolates using a boiling method. All isolates were evaluated for the presence of the Shiga...
toxin-producing genes (stx1, stx2) using PCR amplification. To amplify stx1 and stx2, we used primers described by DesRosiers et al. (2001) and Manna et al. (2006). PCR amplification of was performed in a volume of 50 μl containing 1 μl of the prepared DNA template; 1 μl (each) primer at 20 pmol, 5 μl of 20 mM magnesium chloride, 1 μl of dNTP (40 mM); 0.2 μl Dream Taq DNA polymerase (0.4 U/μl) (Thermo Scientific, Fermentas International Inc., USA) and 40.8 μl molecular grade water. Each reaction volume was processed using a Thermo-cycler (2720 Thermal cycler; Applied Biosystems, USA) at 95°C for 3 min (initial denaturation), followed by 35 cycles at 95°C for 30 s (denaturation), annealing temperature (specific for each primer) for 40 s (binding), and at 72°C for 1 min (extension). The final extension was performed at 72°C for 8 min. We used an aliquot of the reaction mixture without template DNA as a negative control. A STEC strain isolated by Gupta et al. (2016) was utilised as a positive control.

2.4 Antimicrobial susceptibility

The antimicrobial susceptibility profile of STEC isolates was determined by the disc diffusion method (Bauer, 1966) following the Clinical and Laboratory Standards Institute’s (CLSI) guidelines (CLSI, 2012). We followed breakpoints for veterinary and human isolates to interpret the antimicrobial susceptibility test since there are no specific breakpoints available for sheep in the CLSI standard. Briefly, Mueller–Hinton agar was prepared according to the manufacturer’s instructions (Oxoid). Then, A 0.5 McFarland standard was prepared by mixing 0.5 ml of 1% BaCl2•2H2O with 99.5 ml of 1% H2SO4. After mixing, the turbidity of this McFarland standard was compared with the bacterial suspension in sterile saline to prepare the final dilution of bacteria. Finally, commercial discs (Oxoid, UK) for 10 antimicrobials were used: amoxicillin (AML; 10 μg), ampicillin (AMP; 10 μg), ceftriaxone (CRO; 30 μg), chloramphenicol (CHL; 30 μg), ciprofloxacin (CIP; 5 μg), doxycycline (DOX; 30 μg), gentamicin (CN; 10 μg), oxytetracycline (OTE; 30 μg), trimethoprim/sulfamethoxazole (SXT; 30 μg), and tetracycline (TET; 30 μg).

The susceptibility pattern was designated as ‘susceptible’, ‘intermediate’ and ‘resistant’ based on the inhibitory zone and compared with the standard chart from the CLSI.

2.5 PCR assays and sequencing of antibiotic resistance genes

STEC carrying antibiotic resistance genes for AMP and sulfonamides might cause potential public health risks due to the zoonotic significance of the bacteria. Therefore, STEC isolates were screened for the presence of three AMR genes – blatem (AMP), sul1 and sul2 (sulfonamides). We considered these genes because sulfonamides and beta-lactams are two of the more commonly used antibiotics in animal husbandry in Bangladesh. We used a uniplex PCR assay for the identification of each resistance gene. PCR amplification was performed using primers and cycle conditions described in the relevant literature (Belaauaj et al., 1994; Sunde, 2005; Chang et al., 2007). The reaction volume for each PCR assay was 50 μl containing similar proportions as described in this article’s ‘detection of Shiga toxin-producing genes’ section.

One amplified and cleaned PCR product for each resistance gene was sequenced bidirectionally using BigDye Terminator v. 3.1 (ThermoFisher Scientific, Waltham, MA, USA) cycle sequencing protocol by Macrogen Co., Korea. The sequences were compared to the nucleotide database in NCBI Genbank using BLAST with default parameter settings (Altschul et al., 1990).

2.6 Statistical analysis

All data were entered into a spreadsheet programme (Excel 2010; Microsoft Corporation). The prevalence of probable STEC with a 95% confidence interval and the difference between variables (age, breed, sex, health status, and previous history of using antibiotics) for the presence of stx1 and stx2 genes in the probable STEC isolates was shown using a χ2 test. All statistical analyses were performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA. ‘https://www.graphpad.com.

3 RESULTS

3.1 Proportion of animals carrying E. coli and STEC

Two hundred bacterial isolates were recovered from faecal samples from 200 sheep. Of the 200 recovered bacteria, by the conventional method, 144 (72%) of the total recovered sheep isolates were suspected as E. coli through their phenotypical characteristics on agar (pink coloured colonies on MacConkey agar). Of the 144 MacConkey-positive isolates, 123 were identified conventionally as E. coli by EMG agar.

PCR results showed 17 isolates positive for one or both stx1 and stx2 virulence genes, that is, 8.5% of the sheep isolates in the study population were positive as STEC. Ten of the 123 E. coli isolates, that is, 5% of the sheep isolates, were positive for the stx1 gene alone. The proportion of isolates that had the stx2 gene was 3%. Only one isolate had both stx1 and stx2 genes. The distribution of stx1 and stx2 genes in the E. coli isolates did not vary significantly between the variables investigated in this study.

3.2 Antimicrobial susceptibility patterns of the STEC isolates

The antimicrobial susceptibility of the 17 STEC isolates that contained at least one Shiga toxin-producing gene (stx1 and stx2) were tested with ten different agents. The numbers of isolates susceptible, intermediately resistant and resistant to the antimicrobials tested are shown in
**FIGURE 1** Antimicrobial resistance pattern in Shiga toxin-producing *Escherichia coli* isolated from sheep in Bangladesh

**TABLE 1** Frequencies of stx1 and stx2 genotypic isolates resistant to the antimicrobials tested

| Antimicrobial | stx1 (n = 10) | stx2 (n = 6) | stx1 + stx2 (1) |
|---------------|---------------|--------------|-----------------|
| CRO           | 2 (20%)       | 2 (33.33%)   | 0               |
| CIP           | 1 (10%)       | 1 (16.67%)   | 0               |
| TET           | 3 (30%)       | 1 (16.67%)   | 1 (100%)        |
| AMP           | 3 (30%)       | 2 (33.33%)   | 0               |
| DOX           | 2 (20%)       | 1 (16.67%)   | 0               |
| SXT           | 5 (50%)       | 3 (50%)      | 0               |
| GNT           | 1 (10%)       | 1 (16.66%)   | 0               |
| CHL           | 2 (20%)       | 0 (0%)       | 0               |
| AML           | 4 (40%)       | 2 (33.33%)   | 1 (100%)        |
| OXT           | 2 (20%)       | 1 (16.67%)   | 1 (100%)        |

Abbreviations: CRO, ceftriaxone; CIP, ciprofloxacin; TET, tetracycline; AMP, ampicillin; DOX, doxycycline; SXT, sulfamethoxazole–trimethoprim; GNT, gentamicin; CHL, chloramphenicol; AML, amoxicillin; and OXT, oxytetracycline.

Figure 1. Of the tested isolates, 47% isolates were resistant to SXT, and 41% were resistant to AML. The antimicrobial susceptibility profiles of stx1 and stx2 genotypes are displayed in Table 1. Five (50%) stx1 genotypes were resistant to SXT, while four (40%) were resistant to AML. Fifty percent of the stx2 genotypes were resistant to SXT. Two (33.33%) stx2-positive isolates were resistant to AMP, AML and CRO, respectively. One isolate harbouring stx1 and stx2 genes was resistant to TE, AML and OTE.

### 3.3 Proportion of STEC isolates carrying resistance genes

PCR for antimicrobial-resistance genes revealed that six (35%) of the 17 STEC isolates carried the *blaTEM* gene. Eight (47%) and 10 (59%) STEC isolates were positive for *sul1* and *sul2* genes, respectively (Figure 2). The clean and curated sequences for *blaTEM*, *sul1* and *sul2* genes were deposited in the nucleotide database in NCBI Genbank under accession no. MN622888, MN401416 and MN401417, respectively. BLAST search detected a high sequence similarity (99–100%) between partially sequenced resistance genes in this study and published *blaTEM*, *sul1* and *sul2* gene sequences (GenBank Accession Number: KJ923008, AB733642 and GU256641, respectively).

### 4 DISCUSSION

The study investigates the molecular characterisation of STEC strains in sheep of Bangladesh and the presence of AMR genes in STEC. The present study is the first study of STEC performed on sheep of Bangladesh to the authors’ knowledge.
FIGURE 2  Results of PCR assay for three antimicrobial resistance genes: blaTEM, sul1 and sul2. (a) blaTEM gene (716 bp) amplicon: lane M, 1 kb plus DNA ladder; lane P, positive control; lane N, negative control; lanes 1–7, blaTEM-positive isolates; (b) sul1 gene (433 bp) amplicon: lane M, 1 kb plus DNA ladder; lane P, positive control, N, negative control; lanes 1–8, sul1-positive isolates; (c) sul2 gene (433 bp) amplicon: lane M, 1 kb plus DNA ladder; lane P, positive control; N, negative control; lanes 1–8, sul2-positive isolates.

The present study observed 8.5% sheep on smallholdings in the studied area as a reservoir of STEC. This prevalence estimate is likely inaccurate given that a small number of farms were sampled, a small number of animals were sampled, and only one isolate per animal was evaluated. Nevertheless, this is more or less similar to our previous studies, Gupta et al. (2018) and Gupta et al. (2016), where we observed that 7 and 6.2% of healthy buffalo and goat in Bangladesh carry STEC. Islam et al. (2015) also reported a similar prevalence (7.5%) of probable EHEC O157 circulating in smallholders’ cattle. However, a higher prevalence rate (16.2%) among healthy goats in Spain was reported by Orden et al. (2003). In Switzerland, a study on sheep recorded that 29.9% of slaughtered sheep carry STEC (Zweifel et al., 2004). Zschock et al. (2000) recorded a 32% prevalence of STEC in German sheep. In contrast, a lower prevalence (0.7%) was reported in the UK (Milnes et al., 2008). Differences observed in the proportion of STEC genotypes in sheep rectal swab samples tested in this study, and others performed elsewhere were possible because of differences in husbandry practices, agro-climatic variations, sampling, methods of detection, breeds and the age of animals (Battisti et al., 2006; Reid et al., 2002).

The STEC strains isolated from sheep in this study were genetically diverse based on their possession of virulent genes. PCR assays showed that 13.8% of E. coli isolates (17 in 123) possess ≥1 of the two virulent genes studied – stx1 and stx2. Wani et al. (2006) showed that 13.45% (37 in 275) isolates harboured at least one virulent gene in Indian goats tested for stx1, stx2, hly and eae. Islam et al. (2015) showed that 5.98% of isolates (31 in 518) from faecal samples of smallholder cattle in Bangladesh carried at least one virulent gene- stx1, stx2 hly. Ten (8.9%) E. coli isolates were positive for Shiga toxin-producing E. coli gene stx1. The percentage of stx1 carriage in E. coli isolated from Bangladeshi Buffalo was similar (Gupta et al., 2018). A slightly higher prevalence (15.9%) of stx1 was observed in Irish sheep (McCarthy et al., 2019). Gupta et al. (2016) and Wani et al. (2006) also detected that a higher proportion of E. coli isolated from goats could harbour the stx1 gene. Seven (3.5%) E. coli-positive samples were tested positive for the presence of stx2 genes by PCR. MaCarthy et al. (2019) reported a higher prevalence of stx2 virulent factor in isolates from Irish sheep. E. coli isolated from Bangladeshi Buffalo and cattle were observed with a similar prevalence of the stx2 gene.

We observed a diverse antimicrobials resistance pattern in STEC isolates in the present study. A microorganism can be multi-drug resistant when it shows resistance to at least one antimicrobial drug in three or more antimicrobial categories (Magiorakos et al., 2012). The occurrence of multi-drug resistance STEC in sheep in the present study is corroborated by other studies (Gupta et al., 2018; Johura et al., 2017). The present study observed a substantial number of STEC resistance to sulfamethoxazole, TE and AMP which was in line with the findings of previous studies (Gupta et al., 2013; Islam et al., 2013). In addition, STEC isolates showed a higher susceptibility to CHL, CN, CIP and DOX. This finding agrees with the study on STEC in buffaloes and goats of Bangladesh (Gupta et al., 2018; Gupta et al., 2013).

Seven isolates showed either intermediate or complete resistance to AMP in the disk diffusion test, six of them were positive for the blaTEM
gene by PCR. Eleven STEC isolates were recorded with either intermediate or complete resistance to SXT, of which ten were positive for the sul1 gene and eight were positive for the sul2 gene. There were minor differences between the number of resistant STEC based on the disk diffusion method and molecular identification. However, molecular detection like PCR of known AMR genes has increased susceptibility and specificity (Sandle, 2016).

Antibiotic-resistant STEC being harboured in sheep, as detected in the present study, might have zoonotic transferenceability through contaminated food, water, mud and manure (Ma et al., 2020). At least transmission of multi-resistance encoding determinants from coliform bacteria of food animal origin to human strains may occur if sanitary regulations are not observed (Oppegaard et al., 2001). These resistant E. coli isolates can also rapidly spread the resistance to other species of bacteria by horizontal gene transfer (HGT) (Lerminiaux & Cameron, 2019).

5 | CONCLUSIONS

This work provides an understanding of the presence of STEC in healthy sheep on smallholdings in Bangladesh. As observed in the present study, STEC strains carrying AMR genes need to consider sheep as a potential source of the antimicrobial-resistant STEC, which could pose an added risk to public health in Bangladesh.

AUTHOR CONTRIBUTIONS

Conceptualisation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, writing – original draft; writing – review and editing: Mukta Das Gupta. Conceptualisation, investigation, methodology, resources, validation: Arup Sen. Methodology: Mishuk Shaha. Methodology: Avijit Dutta. Conceptualisation, data curation, formal analysis, methodology, resources, software, supervision, visualisation, writing – original draft; writing – review and editing: Ashutosh Das.

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CONFLICT OF INTEREST

The authors have no conflict of interest

ETHICS STATEMENT

No ethical approval was obtained because this study did not contain patient data and only involved non-invasive procedures, that is, faecal samples. However, the animals were handled with care by experienced veterinary practitioners during sample collection. In addition, informed consent through verbal communication was taken from all farm owners before sample collection, assuring that sheep would not be harmed during sampling.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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