Article

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

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Abstract: Inappropriate antimicrobial treatment can pose a risk for developing resistance against antimicrobial drugs in bacteria. Close human contact might have a higher chance of being transmitted to humans from sheep if the sheep population is a potential reservoir of zoonotic pathogens such as shiga toxin-producing *Escherichia coli* (*E. coli*) (STEC). Therefore, this study aimed to examine the sheep population in rural Bangladesh for antimicrobial resistant STEC. We screened 200 faecal samples collected from sheep in three Upazila from the Chattogram district. 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 genes- *bla*TEM, *sul1* and *sul2*. In total, 123 of the 200 tested samples were confirmed positive *E. coli* by cultured based methods. PCR results show 17(13.8%) *E. coli* isolates harboured ≥ one virulent gene (*stx1* or/and *stx2*) of STEC. Six of the tested STEC isolates exhibited *bla*TEM gene; eight STEC isolates had *sul1* gene, and *sul2* gene was detected in ten STEC isolates. To our knowledge, this study is the first to reveal a significant proportion of STEC isolated from sheep in rural Bangladesh harbouring antimicrobial resistance genes.

Keywords: Sheep; *E. coli*; shiga-toxin; antimicrobial resistance genes

1. Introduction

The use of antimicrobial drugs in food-producing animals can lead to antimicrobial resistance (AMR) in foodborne pathogens. AMR in pathogens can be monitored by resistance in the key species of AMR, *E. coli* [1]. *E. coli* is an asymptomatic intestinal inhabitant in food-producing animals. Some *E. coli* variant can cause diarrhoeal disease and extra-intestinal infections by expressing several specific virulence factors, are known as pathogenic *E. coli* [2-4].

Shiga toxin-producing *E. coli* (STEC) is a pathogenic *E. coli* strain which can cause severe illness in human including life-threatening hemolytic uremic syndrome (HUS) [5]. Approximately three-quarters of reported STEC infections in human is attributed to domestic ruminant [6]. Cattle and other ruminants such as sheep and goat are considered the most common reservoirs of STEC. This pathogen can be transmitted to human via direct contact with infected animal, but foods (meat, milk and beverages or water) contaminated with fecal material is considered as the main route of transmission [7-10].

Pathogenic strains of *E. coli* are becoming increasingly resistant by acquiring resistance genes derived from conjugative plasmids. *E. coli* can spread this resistance to other enteric bacteria such as salmonella and campylobacter [11]. In recent years, a widespread resistance to various antimicrobial in *E. coli* reported in humans and non-human sources [12]. In addition, the recent focus on 'One Health' aspects of AMR, surveillance for antimicrobial resistant *E. coli* in animal become more crucial to explore the extend of

public health risk posed by pathogens of zoonotic origin. Monitoring of the occurrence of AMR in STEC in healthy animal may measure the spread of resistant strains and resistance genes from animal to humans, hence, crucial for revise and implementation of new control measures for antibiotic resistance.

In Bangladesh, previous studies reported the prevalence of STEC in various livestock species [13-16]. However, there is no report on the prevalence of STEC in sheep. In addition, there is no Bangladeshi study investigate the antibiotic resistance at gene level. Taken together the current study aimed to determine the antibiotic resistance genes and explore the prevalence of STEC from fecal samples of healthy sheep in Bangladesh. *E. coli* isolates were screened for the presence of major virulence genes: *stx1* and *stx2* and the presence of three antimicrobial resistance genes- *bla*TEM, *sul1* and *sul2* were determined.
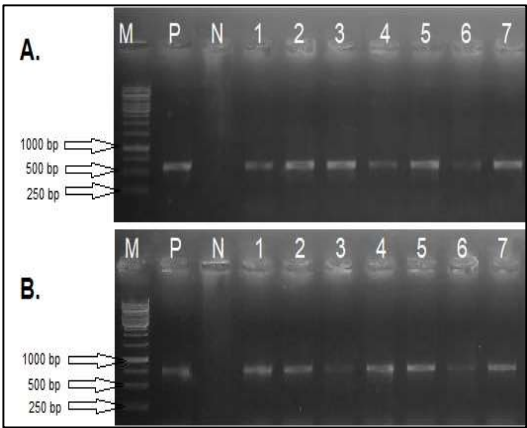
2. Results

2.1 Prevalence of and STEC

A total of 200 bacterial isolates were recovered from sheep faecal samples. From the 200 recovered bacteria, by the conventional method, 144 (72%) of the total recovered 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 confirmed conventionally as *E. coli* by EMB agar. Among the total 123 *E. coli* isolates, 17 (13.8%) were characterized by PCR as having one or both *stx1*, *stx2* virulence genes. Therefore, the prevalence of STEC isolates that carried at least one virulence gene in the total study population could be 8.5% (95% CI 5.3 to 13.3).

2.2 Diversity of identified STEC

PCR results of some isolates representing amplicons of *stx1* and *stx2* genes are depicted in Figure 1. PCR results showed that 10 (8.9%) isolates were positive for the *stx1*, and 6 (5.69 %) of the *E. coli* isolates possessed only the *stx2* gene. One isolate was found positive for both *stx1* and *stx2* genes. The prevalence of STEC possessing the virulent gene *stx1* and *stx2* was 5.0 % (95% CI 2.63 to 9.07) and 3.0 % (95% CI 1.23 to 6.54) respectively in the study population. Only one isolate [0.5%, (95% CI 0.00.01 to 0.306)] possessed the combination of both *stx1* and *stx2* genes.

Figure 1. Results of PCR assay for two shiga toxin producing genes: *stx1* and *stx2*. A. *stx1* gene (614 bp) amplicon: lane M=1 kb plus DNA ladder, lane P, Positive control, lane N, Negative control, lanes 1–7, *stx1*-positive isolates; B. *stx2* gene (779 bp) amplicon: lane M= 1 kb plus DNA ladder; lane P, Positive control, N=Negative control, lanes 1–7, *stx2*-positive isolates.

The prevalence of STEC isolates possessing the *stx1* gene was not varied between the variables – age, breed, sex, health status, and previous history of using any antibiotics. However, a higher isolation rate is observed in adult than in lamb (P=0.3572) (Table S1), although the isolation rate was not statistically significant. Male had a relatively higher isolation rate of *stx2* genotype than female sheep, but statistically insignificant (P=0.4726). The prevalence of *stx2* genotype in sheep population not varied significantly between variables studied, but a higher isolation rate was observed in sheep reared in the free-range system than semi-intensive system and fair than healthy sheep (Table S2).

2.3 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 to 10 different agents. The numbers of isolate sensitive, intermediately resistant and resistant to the antimicrobials tested are shown in Figure 2. Of the tested isolates, 82%, 76%, 71% and 71% were sensitive to chloramphenicol, gentamicin ciprofloxacin and ampicillin, respectively; 47% isolates were resistant to trimethoprim-sulfamethoxazole, and 41% were resistant to amoxicillin.
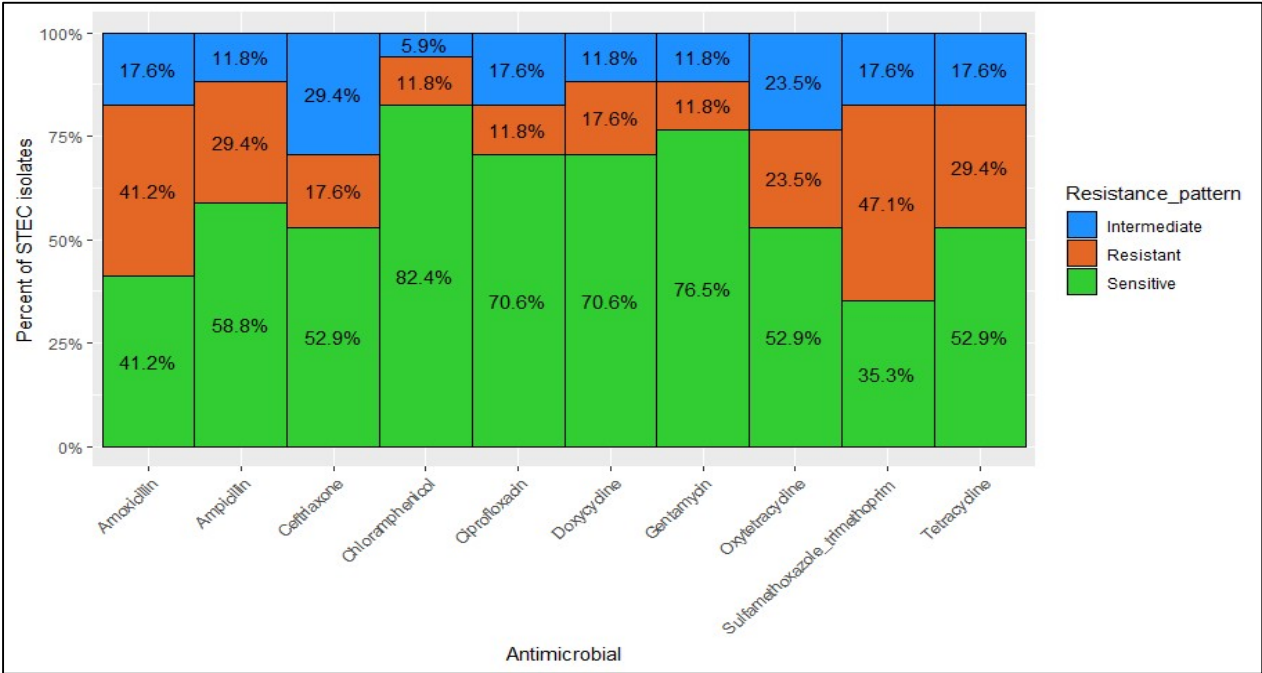

Figure 2. Antimicrobial resistance pattern in shiga toxin-producing *Escherichia coli* isolated from sheep in Bangladesh.

The antimicrobial susceptibility profiles of *stx1* and *stx2* genotypes are displayed in Table 1. Five (50%) and 4 (40%) of the *stx1* genotypes tested were resistant to trimethoprim-sulfamethoxazole and amoxicillin, respectively; 50% of the *stx2* genotypes were resistant to trimethoprim-sulfamethoxazole. Ampicillin, amoxicillin and Ceftriaxone were resistant to 33.33% of *stx2* positive isolates. One isolate that harboured *stx1* and *stx2* genes was 100% resistant to tetracycline, amoxicillin, and oxytetracycline.

Table 1. Frequencies of *stx1* and *stx2* genotypic isolates of *Escherichia coli* resistant to the antimicrobials tested

Antimicrobial	No. resistant isolates (%)		
	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
DOC	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%)

CRO, Ceftriaxone; CIP, Ciprofloxacin; TET, Tetracycline; AMP, Ampicillin; DOC, Doxycycline; SXT, Sulfamethoxazole-Trime-thoprim; GNT, Gentamycin; CHL, Chloramphenicol; AML, Amoxycillin and OXT, Oxytetracycline.

2.4 Proportion of STEC isolates carrying resistance genes

PCR for antimicrobial-resistance genes revealed six (35%) of the 17 STEC isolates carried *bla*TEM gene. Eight (47%) and 10 (59%) STEC isolates were positive for *sul1* and sul2 genes, respectively (Figure 3). The clean and curated sequences for *bla*TEM, *sul1* and *sul2* genes were deposited in nucleotide database in NCBI Genbank under accession no. MN622888, MN401416 and MN401417, respectively. BLAST search detected a high sequence similarity (99-100%) between the partially sequenced resistance genes in this study and published *bla*TEM, *sul1* and *sul2* gene sequences (GenBank Accession Number: KJ923008, AB733642 and GU256641, respectively).
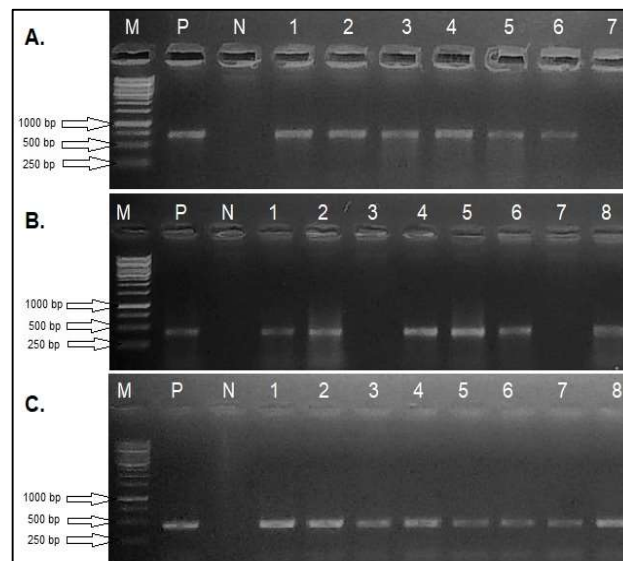
Figure 3. Results of PCR assay for three antimicrobial resistance genes: *bla*TEM, *sul1* and *sul2*. A. *bla*TEM gene (716 bp) amplicon: lane M=1 kb plus DNA ladder, lane P, Positive control, lane N, Negative control, lanes 1–7, *bla*TEM-positive isolates; B. sul1 gene (433 bp) amplicon: lane M= 1 kb 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.

3. Discussion

The study investigates the molecular characterization of STEC strains in sheep of Bangladesh and the presence of antimicrobial resistance genes in STEC. This is the first longitudinal study of STEC performed on sheep of Bangladesh to the authors' knowledge. The results of the study revealed that out of 200 samples, all the 144 isolates, as evidenced by the production of large pink-coloured colonies (a selective medium recommended for E. coli), were probably not *E. coli* since only 123 were found to be *E. coli* as they produced metallic green sheen on EMB agar.

The present study observed 8.5 % sheep on smallholding in Bangladesh could be reservoir of STEC. This is more or less similar to the findings of our previous studies, Gupta et al., (2018) and Gupta et al., (2016), where we observed 7% and 6.2% of healthy buffalo and goa in Bangladesh carry STEC [13, 14]. Islam et. al., (2015) also reported a similar prevalence (7.5%) of probable EHEC O157 circulating in smallholders' cattle [16]. However, a higher prevalence rate (16.2%) among healthy goat in Spain was reported by Orden et al. [17]. A study on sheep in Switzerland recorded 29.9% of slaughtered sheep carry STEC [18]. Zschock et al. (2000) recorded a 32% prevalence of STEC in German sheep [19]. In contrast, lower prevalence was reported in UK and Saudi Arabia [20, 21]. Differences observed in the proportion of STEC genotypes in sheep rectal swab samples tested in this study and others performed elsewhere were possibly because of differences in husbandry practices, agro-climatic variations, sampling, methods of detection, breeds and the age of animals [22, 23]. However, proportions of sheep carrying STEC between the different variables did not differ significantly. Similar husbandry practice for sheep in sampled smallholdings might be the reason for this.

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% *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 goat tested for *stx1*, *stx2*, *hly* and *eae* [24]. Islam et al., (2015) showed that 5.98% of isolates (31 in 518) from faecal samples of smallholders' cattle in Bangladesh carried at least one virulent gene- *stx1*, *stx2* and *hly* [16]. 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 also found similar (Gupta et al., 2018). A slightly higher prevalence (15.9%) of *stx1* was observed in Irish sheep [25]. Gupta et al. (2016) and Wani et al. (2006) also detected that a higher proportion of *E. coli* isolated from goat 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 [25]. *E. coli* isolated from Bangladeshi Buffalo and cattle observed with a similar prevalence of the *stx2* gene. However, goat isolates in Bangladesh carry more stx2 than sheep isolates in the present study [13].

In the present study, we observed a diverse antimicrobials resistance pattern in STEC isolates. The occurrence of Multidrug resistance (MDR) STEC in sheep in the present study is corroborated by other studies [14, 26]. The finding is indicative of a selective pressure of several groups of antimicrobial substances on the farm related to treatment sometime in the past. The present study observed a higher rate resistance of STEC to sulfamethoxazole, tetracycline, oxytetracyclin, ampicillin and amoxicillin, an agreement with some previous reports [27, 28]. STEC isolates showed a higher sensitivity to chloramphenicol, gentamycin, ciprofloxain and doxycyclin. This finding agrees with the study on STEC in buffaloes and goat of Bangladesh [14, 27]. These observations warrant more clinical trials to identify whether chloramphenicol, gentamycin, ciprofloxain and doxycyclin are the choice of antibiotics in the control of STEC infection in animals and human in Bangladesh.

Seven isolates showed either intermediate or complete resistance to ampicillin in disk diffusion test, six of them were positive for BLATEM gene by PCR. Eleven STEC isolates were recorded with either intermediate or complete resistance to sulfamethoxazole-trimethoprim, of which 10 were positive for sul1 gene and 8 were positive for sul2 gene. There were minor differences between the number of resistant STEC based on disk diffusion method and molecular identification. However, molecular detection of known antimicrobial resistance genes considered to have increased sensitivity and specificity [29].

The detection of antibiotic resistance genes in the present study signifies that there is a risk of spread of the antimicrobial resistance to human by direct or indirect transmission of resistant isolates through food, water, mud, and manure [30]. At least of a transmission of multi-resistance encoding determinants from coliform bacteria of food animal origin to human strains, may take place, if sanitary regulations are not observed [31]. These resistant *E. coli* isolates can also rapidly spread the resistance to other species of bacteria by horizontal gene transfer (HGT) [32].

4. Materials and Methods

4.1 Sample collection, Culture and isolation

A total of 200 fecal samples were collected from sheep from three upazilas (a upazila is a subdistrict level area) of Southeastern Bangladesh. The samples were collected from apparently healthy sheep of all ages, sexes, and breeds on smallholder farms. Fecal materials from each animal were collected by inserting a sterile cotton swab into recto-anal junction. After collection, swabs were dipped into buffered peptone water and stored in an icebox (4oC). Collected swabs were shipped immediately to the Microbiology and Veterinary Public Health Laboratory, Chittagong Veterinary and Animal Sciences University.

A presumptive *E. coli* isolate was identified based on the growths in selective media, as described previously [14, 33]. 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 incubated at 37 °C for 24 h. Five well isolated bright pink colonies MacConkey agar subcultured 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 and five cross-sectional colonies from homogenous pink colonies were 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% glycerin until assessment for virulence genes.

4.2 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. The details of primers and PCR products are presented in Table 2. PCR amplification of was performed in a volume of 50 μl containing1 μl of the prepared DNA template; 1 μl (each) primer at 20 pmol, 5 μl of 20mM magnesium chloride, 1 μl of dNTP (40 nM), 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 sec (denaturation), annealing temperature (specific for each primer) for 40 sec (binding), and at 72°C for 1

min (extension). Final extension was performed at 72°C for 8 min. An aliquot of the reaction mixture without template DNA was used as a negative control. A STEC strain isolated by Gupta et al, (2016) [13] was utilized as a positive control.

Table 2. Oligonucleotide primer sequences for *stx1* and stx2 genes, annealing temperature, size of the PCR product, and reference of each primer used.

Primer	Primer Sequence (5'– 3')	Target gene	Annealing temp. (°C)	Amplicon (bp)	References
stx1 F	ACA CTG GAT GAT CTC AGT GG	*stx1*	58	614	[34]
stx1 R	CTG AAT CCC CCT CCA TTA TG				
stx2 F	CCA TGA CAA CGG ACA GCA GTT	stx2	58	779	[35]
stx2 R	CCT GTC AAC TGA GCA GCA CTT T				

4.3 Antimicrobial susceptibility

Antimicrobial susceptibility profile of STEC isolates was determined by disc diffusion method [36] following Clinical and Laboratory Standards Institute's (CLSI) guidelines. Commercial discs (Oxoid, UK) for ten 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 (TE, 30 µg).

The susceptibility pattern was designated as "sensitive", "intermediate" and "resistant" based on inhibitory zone and comparing with the standard chart from the CLSI.

4.4 PCR assays and sequencing of antibiotic resistance genes

STEC isolates were screen for the presence of three antimicrobial resistance gene-blaTEM (ampicillin), *sul1* and *sul2* (sulfonamide). Uniplex PCR assay was used for the identification of each resistance gene. PCR amplification was performed using primers and cycle conditions described in the relevant literature (Table 3). The rection volume for each PCR assay was 50 µl containing similar proportions as described in the "detection of Shiga toxin-producing genes" section of this article.

One amplified and cleaned PCR product for each resistance gene was sequenced bi-directionally using BigDye Terminator v. 3.1 (ThermoFisher Scientific, Waltham, MA, USA) cycle sequencing protocol by Macrogen Co., Korea. To detect sequence similarity, the sequences were compared to the nucleotide database in NCBI Genbank using BLAST [37].

Table 3. Oligonucleotide primer sequences for *blaTEM*, *sul1* and *sul2* genes, annealing temperature, size of the PCR product, and reference of each primer used.

Primer	Primer Sequence (5'– 3')	Target gene	Annealing temp. (°C)	Amplicon (bp)	References
blaTEM F	TAC GAT ACG GGA GGG CTT AC	*blaTEM*	60	716	[38]
blaTEM R	TTC CTG TTT TTG CTC ACC CA				
sul1 F	CGG CGT GGG CTA CCT GAA CG	*sul1*	51	433	[39]
sul1 R	GCC GAT CGC GTG AAG TTC CG				
sul2 F	CCT GTT TCG TCC GAC ACA GA	*sul2*	59	435	[40]
sul2 R	GAA GCG CAG CCG CAA TTC AT				

4.5 Statistical analysis

All data were entered into a spreadsheet programme (Excel 2010, Microsoft Corporation). The prevalence of probable STEC with 95% confidence interval and the difference between variables for the presence of *stx1* and *stx2* genes in the probable STEC isolates was shown using a $\chi 2$ test. All statistical analyses were performed by using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com".

5. Conclusions

In our knowledge, this study provides first evidence that a significant proportion of STEC isolated from healthy sheep on smallholdings in Bangladesh carrying antimicrobial resistance genes. This finding would be useful in advocating any future approach targeting source-mitigation of zoonotic pathogens to protect the public health in rural Bangladesh.

Supplementary Materials: Table S1: Variable-wise prevalence of *stx1* genotype of STEC isolated from sheep in Bangladesh, Table S2: Variable-wise prevalence of *stx2* genotype of STEC isolated from sheep in Bangladesh.

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