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ORIGINAL ARTICLE

Isolation, molecular characterization and antibiotic resistance of Shiga Toxin–Producing *Escherichia coli* (STEC) from buffalo in India

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Significance and Impact of the Study: The buffaloes from different districts of West Bengal, India, are important reservoir of multidrug-resistant Shiga toxin–producing *Escherichia coli* (STEC). India is home to more than 56% of world buffalo population, traditionally raised by farmers. So, there is a major risk of transmission of STEC among the human population of this part of the globe. However, there is no prevalence study of STEC from healthy or diarrhoeic buffalo in India. The present study reports for the first time in India about isolation, molecular characterization and antibiotic resistance pattern of STEC in healthy buffaloes.

Keywords
antibiotic resistance, buffalo, India, Shiga toxin–producing *Escherichia coli*, West Bengal.

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Abstract

In total, 363 *Escherichia coli* were isolated from 165 faecal samples of healthy buffaloes in West Bengal, India. Twenty-four of these isolates (6·61%) were found to carry at least one gene characteristic for Shiga toxin–producing *Escherichia coli* (STEC). These STEC strains belonged to 13 different O-serogroups. The stx_1 gene was present in 23 (95·8%) of total STEC isolates, whereas 20 (83·3%) STEC isolates carried the gene stx_2. Twelve strains of *E. coli* (50% of total STEC isolates) possessed enterohaemolysin (*ehxA*) gene in combination with others. Fourteen (58·33%) isolates found to possess *saa* gene. However, no *E. coli* was detected harbouring gene for intimin protein (*eaeA*). Of 23 stx_1-positive isolates, seven (30·43%) were positive for genes of the stx_{1C} subtype. Of the 20 isolates with the stx_2 gene, 25% (5/20) possessed stx_{2C} and 10% (2/20) possessed stx_{2d} gene. The phylogenetic analysis after RAPD of STEC strains revealed six major clusters. The isolated STEC strains were resistant most frequently to erythromycin (95·83%), cephalothin (62·5%), amikacin (54·17%), kanamycin (45·83%) and gentamicin (41·67%) group of antibiotics. No ESBL-producing (bla_{CTXM}, bla_{TEM}, bla_{SHV}) or quinolone resistance gene (*qnrA*) was detected in the STEC isolates.

Introduction

Escherichia coli are facultative anaerobic bacteria and normal habitat of lower intestine in man and animals, although some toxin producing strains can produce pathogenicity ranging from mild diarrhoea to fatal complications. One of such important toxigenic groups is Shiga toxin–producing *Escherichia coli* (STEC). The clinical illness includes a spectrum of human sufferings like bloody diarrhoea and even life-threatening conditions such as haemolytic uraemic syndrome (HUS) (Paton and Paton 1998a). In addition to the enterohaemorrhagic *E. coli* O157:H7 (EHEC, a subgroup of STEC), which received world wide 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; Espié *et al.* 2008; King *et al.* 2009). This scenario becomes graver during infection with antibiotic-resistant strains of STEC due to poor response or lack of response to the applied treatment.

The ability of STEC to cause human disease is due to the production of Shiga toxins, which inhibit protein synthesis of the host cells leading to cell death (Karmali *et al.* 2010). These toxins are subdivided into two major groups: Shiga toxin 1 (stx1) and Shiga toxin 2 (stx2). Stx1 is a rather homologous group in which three variants (stx1, stx1c and stx1d) have been described (Brett *et al.* 2003). The stx2 group is more heterogeneous and is comprised of several subtypes (stx2c, stx2d, stx2e, stx2f, stx2 g and activatable stx2d) (Leung *et al.* 2003). Apart from Shiga toxin, STEC strains may possess other virulence factor necessary for infection such as intimin, encoded by the chromosomal gene *eaeA*, which is found on a pathogenicity island termed as the locus of enterocyte effacement (LEE) (Kaper *et al.* 2004). Additional virulence factors of STEC include enterohaemolysin, a pore-forming cytolysin on eukaryotic cells (Schmidt *et al.* 1995), bifunctional catalase peroxidase (katP) (Brunder *et al.* 1996) and the *etpD* gene cluster, encoding a type II secretion pathway (Schmidt *et al.* 1997). Several proteins were proposed to be novel adhesion factors of STEC like ToxB (a protein identified from the large, 93-kb plasmid pO157 and required for full expression of adherence of O157: H7 strain), and saa (STEC autoagglutinating adhesin identified in the LEE-negative strains) (Toma *et al.* 2004).

Treatment for STEC infection with antimicrobials is controversial due to increased *in vivo* production of the Shiga toxins with the applied antibiotics (Paton and Paton 1998a). However, a few reports of antibiotic resistance in STEC are available (Schroeder *et al.* 2002). Extended-spectrum β-lactamases (ESBL), produced by *E. coli*, offered resistance against higher generations of cephalosporins (except cephamycins and carbapenems) and monobactam. Currently, ESBL-producing organisms are becoming a major threat for patients in the hospital and community. There are three major classes of ESBL (TEM, SHV and CTX-M) encoded by $bla_{TEM,}$ bla_{SHV} and $bla_{CTX\text{-}M}$ genes, respectively (Geser *et al.* 2012).

Shiga toxin–producing *Escherichia coli* infections are food-borne zoonoses transmitted through the consumption of contaminated food such as minced beef, raw milk and unpasteurized dairy products, because the main reservoir of STEC appears to be ruminants (Normanno 2007). Domestic ruminants like cattle, sheep and goats have been established as major natural reservoirs for STEC and play a significant role in the epidemiology of human infections (Karmali *et al.* 2010). Buffaloes as STEC reservoir have been detected in some European countries (Conedera *et al.* 2004; Galiero *et al.* 2005; Oliveira *et al.* 2007; Seker and Yardimci 2008; Lorusso *et al.* 2009) as well as a few Asian countries like Srilanka, Bangladesh and Vietnam (Mohammad *et al.* 1986; Islam *et al.* 2008; Vu-Khac and Cornick 2008). However, to the author's best knowledge, there is no such report from India, although India is home to more than 56% of world buffalo population, traditionally raised by farmers for milk and meat production or working in field or farm. So a need of systemic study for the prevalence of STEC, their genotype, phylogenetic relationship, antibiotic resistance from Indian buffaloes was felt.

Results and discussion

Isolation of *Escherichia coli* from faecal samples of buffaloes

Indian dairy sector including the buffaloes is purely domestic, not maintained in the intensive farm system as in developed countries. So, there is a major risk of transmission of the zoonotic pathogens like Shiga toxin–producing *Escherichia coli* (STEC) among the human population of this part of the globe. However, there was no systematic study regarding this aspect in healthy or diarrhoeic buffalo in India. Therefore, the present study was conducted to determine the STEC occurrence, their virulence gene profile, phylogenetic relationship and antibiotic resistance pattern in buffalo from seven districts (Bankura, North 24 parganas, Murshidabad, Maldah, Hooghly, Nadia & Burdwan) districts of West Bengal, India.

From the 165 faecal samples of apparently healthy buffaloes examined, a total of 363 isolates were identified as *E. coli* on the basis of characteristic pink-coloured colonies on MacConkey agar, metallic sheen in EMB agar, Gram-negative small rod appearance in stained smear, and biochemically they were catalase (+ve), oxidase (−ve), Indole-Methyl Red-Voges Proskauer-Citrate (+ + − −) and urease (−ve), characteristic of standard *E. coli* strains.

Serogrouping of *Escherichia coli* isolates

Of the 363 strains of *E. coli* isolated from buffalo, 77 (21·21%) were untypeable, 21 (5·79%) were rough and remaining 265 strains were typed into 52 different serogroups. O120 serogroup was isolated from largest number of buffalo (11·7%), followed by O25 (10·6%), O60 (9·06%) and O85 serogroup (7·17%). In contrast, Oliveira *et al.* (2007) most frequently isolated O74 serogroup as STEC from buffalo in Brazil. It has been found that eight serogroups namely O22, O25, O41, O60, O76, O85, O112

and O120 constituted 55% of the *E. coli* isolates in this study. We have also found that only the serogroups O25, O60 and OUT were present in isolates from all the studied seven districts of West Bengal, India. No report of *E. coli* isolation from buffalo in West Bengal or India was available to compare the result.

Detection of STEC by PCR

Escherichia coli harbouring any gene for Shiga toxins (stx_1 or stx_2) was detected in 24 (6·61%) of the 363 *E. coli* isolates. Similar occurrence rate of STEC (5·4%) was also found in water buffalo calves reared in Italy (Galiero *et al.* 1997), although Mohammad *et al.* (1985), Oliveira *et al.* (2007), Islam *et al.* (2008) and Vu-Khac and Cornick (2008) found higher prevalence rate of STEC in buffalo (28, 0–64·3, 37·9 and 27%, respectively) in other countries.

These STEC strains belonged to 13 different serogroups (O2, O5, O20, O22, O25, O41, O44, O60, O76, O85, O87, O113 and O120) and two were untypeable (OUT) (Table 1). STEC has been identified from five of seven districts studied except in North 24-parganas and Burdwan in West Bengal, India. Similarly, Mohammad *et al.* (1986) also found O5, O20 and O76 serogroup as STEC from diarrhoeic cattle and buffalo calves, and O41, O76 and untypeable serogroups were also observed as STEC isolated from buffalo in Bangladesh (Islam *et al.* 2008). In addition, O22, O113 and untypeables harbouring the genes for STEC were isolated from healthy buffaloes in Brazil (Oliveira *et al.* 2007). Certain serogroups recovered in the present study like O2, O85, O113 were earlier recorded to be associated with human infection and therefore constitute a significant risk of public health hazard (Constantiniu 2002).

Subtyping of *stx* genes by PCR

The stx_1 gene was present in 23 (95·8%) of total STEC isolates, whereas 20 (83·3%) STEC isolates carried the gene stx_2. The present finding is in agreement with the previous study by Islam *et al.* (2008) where stx_1 gene was found in a larger number than stx_2 of non-O157 STEC isolates from buffalo in Bangladesh, geographically nearer to West Bengal state of India in comparison with other studied countries, whereas in other countries, Galiero *et al.* (2005), Oliveira *et al.* (2007) and Vu-Khac and Cornick (2008) found a larger number of stx_2-positive isolates from buffalo than stx_1.

Characterization of these 23 *E. coli* strains possessing the stx_1 gene showed that 30·43% (7/23) of these isolates were positive for genes of the stx_{1C} subtype. In contrast, Vu-Khac and Cornick (2008) found a larger number of stx_{1C} possessing STEC isolates (65·63%; 42/64) from buf-

Table 1 Genotypic pattern, phylogenetic clusters and serogroups of isolated Shiga toxin–producing *Escherichia coli* from buffalo in different districts of West Bengal, India

Sl. No.	Genotype	Serogroup	Source district	Annotation used in the dendogram
1.	$Stx_{1C}stx_{2C}ehx_{A}saa$	O113	Bankura	1-C
2.	$Stx_{1C}stx_{2C}ehx_{A}saa$	O113	Bankura	2-B
3.	$Stx_{1C}stx_{2}ehx_{A}saa$	OUT	Bankura	1-G
4.	$Stx_{1}stx_{2}ehx_{A}saa$	O60	Bankura	2-G
5.	$Stx_{1}stx_{2}ehx_{A}$	O25	Bankura	1-F
6.	$Stx_{1}stx_{2}$	OUT	Murshidabad	2-C
7.	$Stx_{1C}stx_{2d}saa$	O41	Murshidabad	1-B
8.	$Stx_{1}ehx_{A}saa$	O60	Murshidabad	2-F
9.	$Stx_{1}stx_{2}ehx_{A}saa$	O85	Hooghly	1-E
10.	$Stx_{1C}stx_{2}saa$	O85	Hooghly	4-A
11.	$Stx_{1}stx_{2}saa$	O25	Hooghly	2-D
12.	$Stx_{1}stx_{2d}saa$	O76	Hooghly	2-E
13.	ehx_{A}	O2	Hooghly	2-A
14.	$Stx_{1C}stx_{2C}ehx_{A}saa$	O41	Maldah	3-A
15.	$Stx_{1}stx_{2}ehx_{A}saa$	O120	Maldah	4-C
16.	$Stx_{1C}stx_{2C}ehx_{A}saa$	O25	Maldah	1-A
17.	$Stx_{1}stx_{2}$	O44	Nadia	3-G
18.	$Stx_{1}stx_{2}$	O120	Nadia	3-C
19.	$Stx_{1}stx_{2}$	O22	Nadia	1-D
20.	$Stx_{1}ehx_{A}saa$	O20	Nadia	3-D
21.	$Stx_{1}stx_{2C}$	O25	Nadia	3-B
22.	Stx_{1}	O5	Nadia	4-B
23.	$Stx_{1}stx_{2}$	O87	Nadia	3-F
24.	$Stx_{1}stx_{2}$	O60	Nadia	3-E

falo in central Vietnam. In our study, most of the stx_{1C}-positive isolates possessed ehx_A, *saa* or both genes. In contrast, Oliveira *et al.* (2007) found all the stx_{1C}-positive strains were devoid of additional virulence factors. As reported in a previous study by Friedrich *et al.* (2003), stx_{1c}-encoding strains are less pathogenic for human.

Of the 20 isolates with the stx_2 gene, 25% (5/20) were positive for stx_{2C}, and 10% (2/20) were positive for stx_{2d} gene. This result is supported by Vu-Khac and Cornick (2008) who also found stx_{2C} as dominant variant (36%) than stx_{2d} (14%) in buffalo.

No *Escherichia coli* harbouring gene for intimin protein, that is, *eaeA,* (Enteropathogenic *E. coli*) was detected in the present study. This is in agreement with other reports like Vu-Khac and Cornick (2008), who could not isolate single *eaeA*-positive *E. coli* strain from buffalo in Vietnam and Oliveira *et al.* (2007) in Brazil, where all STEC isolates from buffalo were devoid of *eaeA* gene. Islam *et al.* (2008) also found that all non-O157 STEC isolates from buffalo lacked *eaeA* gene. Intimin gene (*eaeA*) is a part of the large pathogenicity island, known as Locus for enterocyte effacement (LEE). Although this LEE appears to confer enhanced virulence, the presence of it is not essential for pathogenesis, because a number of cases of severe STEC disease, including HUS, as well as occasional outbreaks, have been caused by the LEE-negative strains (Paton *et al.* 1999; Lepšanović *et al.* 2012). The presence of the LEE is associated with certain O serogroups like O26, O103, O111, O145 and O157 (Sandhu *et al.* 1996); none of which has been identified as STEC in the present study.

Twelve strains of *E. coli* (50% of total STEC isolates) possessed enterohaemolysin (*ehxA*) gene in combination with others. However, only one strain of *E. coli* belonged to O2 serogroup was detected harbouring only enterohaemolysin gene, that is, *ehxA* (Table 1). Higher occurrence of *ehxA* gene was observed by Oliveira *et al.* (2007) and Vu-Khac and Cornick (2008) who found 78·9 and 81%, respectively, in the *E. coli* isolates from buffalo, but Islam *et al.* (2008) detected only 6·1% *ehxA* in the non-O157 STEC isolates in buffaloes.

Paton *et al.* (2001) first described a novel plasmid-encoded adhesin, known as saa (STEC autoagglutinating adhesin) in *eaeA*-negative *E. coli* strain responsible for an outbreak of HUS. The *saa* gene was present in 14 (58·33%) isolates from buffalo in this study, out of which 10 (71·43%) were associated with the ehx_A gene. This is in contrast to the previous studies by Oliveira *et al.* (2007), Vu-Khac and Cornick (2008) and Islam *et al.* (2008) where *saa* gene was detected in 83·5, 78 and 8·1% *Escherichia coli* isolates from buffaloes, respectively. However, obtaining *saa* gene in *eaeA*-negative strains and association with *ehxA* gene is in agreement with the findings of other worker (Jenkins *et al.* 2003).

RAPD analysis of STEC isolates

All 24 STEC strains were characterized by RAPD-PCR to determine the genetic diversity and phylogenetic relationship among the strains. All the strains were typeable with primer 1247, and they produced amplified fragment ranging from 291 to 1888 bp. The phylogenetic analysis of STEC strains (analysed by Doc-itLs image analysis software, UVP, Cambridge, UK) after RAPD reveals six major clusters (A–F) (Fig 1). Even though the RAPD banding patterns were not identical, the phylogenetic analysis found similarity between many isolates of STEC from different districts of West Bengal, India. Except for cluster F, the rest were comprised of STEC strains from different districts. The two isolates in the cluster F belonged to same serogroup (O113). This is a support to the observation by Krüger *et al.* (2006) where he found many RAPD pattern among the non-O157 STEC isolates belonging to the same serotypes, although no district-specific cluster was observed.

Screening for other genes like *katp, etpD* and *toxB* in STEC isolates by PCR

In this study, it has been observed that none of the isolates possessed *toxB, katP* and *etpD* genes, which are in agreement with Islam *et al.* (2008).

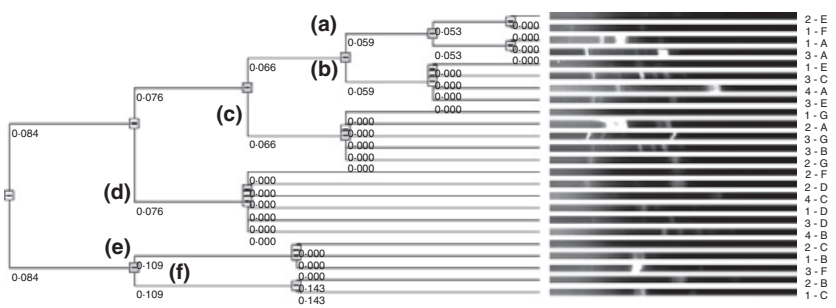

Figure 1 Phylogenetic analysis of Shiga toxin–producing *Escherichia coli* (STEC) strains isolated from healthy buffalo in West Bengal (India). The neighbour-joining method was used to summarize the similarity of RAPD-PCR profiles of STEC strains in a dendogram containing six major clusters (a–f).

Antibiotic sensitivity test of STEC isolates

All the STEC isolates were tested for resistance against 26 antimicrobial agents. Resistance was observed most frequently to erythromycin (95·83%), cephalothin (62·5%), amikacin (54·17%), kanamycin (45·83%), gentamicin (41·67%) and less frequently to chloramphenicol (4·17%), norfloxacin (4·17%), amoxicillin (4·17%) and co-trimoxazole (4·17%). No resistance was observed in case of ceftazidine, levofloxacin, cefepime along with tazobactam. Multidrug resistance was observed in nine (37·5%) STEC isolates. For azithromycin, streptomycin and neomycin, although none of the strains were sensitive to those antibiotics, 70·83, 95·83 and 75% strains showed intermediate susceptibility. Multidrug resistance was observed in nine STEC strains. Oliveira *et al.* (2007) found 17·8% resistance to streptomycin and ampicillin among the STEC isolated from water buffalo in Brazil. In India, Khan *et al.* (2002a) observed resistance to one or more antibiotics in 49·2% of the STEC strains from cattle, and some of the strains exhibited multidrug resistance. Arya *et al.* (2008) also found 100% resistance against kanamycin and cephalexin followed by cephaloridine, enrofloxacin, amikacin, ampicillin, tetracycline, ceftiofur, ciprofloxacin, colistin and co-trimoxazole in STEC isolated from diarrhoeic calves in Gujarat, India. This kind of multidrug-resistant STEC strains poses serious threat to human health by affecting treatment against it. In addition, this resistant bacterial pool in the intestine of buffalo may transfer the responsible gene to other related pathogens and make them also multidrug resistant.

Detection of ESBL-producing and quinolone resistance genes in STEC isolates

None of the STEC isolates possessed the genes for ESBL production (bla_{CTXM}, bla_{TEM}, bla_{SHV}) and quinolone resistance (*qnrA*). Although food animals are one of the important sources of ESBL-producing *E. coli* in several countries in Europe (Geser *et al.* 2012) and Asia (Hiroi *et al.* 2012), there are very limited reports of STEC isolates harbouring ESBL gene (Valat *et al.* 2012).

Materials and methods

Sampling

A total 165 faecal samples of buffaloes were collected randomly from seven districts (Bankura, North 24 parganas, Murshidabad, Maldah, Hooghly, Nadia & Burdwan) of West Bengal, India. The samples were collected from apparently healthy buffalo of all age, sex and breed during the period of October 2009 to January 2010.

Isolation of *Escherichia coli* from faecal samples

Faecal samples were collected into sterile vials directly from rectum by sterile cotton swab stick (HiMedia, Mumbai, India). All the samples collected were placed in ice in a thermo flask and were immediately brought to the laboratory for further examination. In the laboratory, the samples were kept in the nutrient broth (HiMedia) and incubated at 37°C for overnight. It was transferred to MacConkey's agar (HiMedia) and again incubated at 37° C for overnight. Next day 2–3 rose pink colonies were randomly picked and transferred to EMB agar (HiMedia) followed by an overnight incubation at 37°C. Colonies were observed for metallic sheen, and single colony was streaked to nutrient agar (HiMedia) slant for further biochemical confirmation. All the pure cultures obtained from nutrient agar slant were subjected to Gram's staining and standard biochemical tests as described by Wani *et al.* 2004.

Serogrouping

All the *Escherichia coli* isolates after confirmation by biochemical tests were sent for O-serogrouping to National Salmonella & Escherichia Centre, Central Research Institute, Kasuli, HP, India.

Bacterial DNA extraction

For PCR-based detection of Shiga toxin genes from all the *E. coli* isolates, DNA was extracted as per the previously described method of Wani *et al.* (2003) with some modifications. Instead of 100 μl, the extraction was performed in 60-μl sterile distilled water.

Detection of Shiga toxin–producing *Escherichia coli* (STEC) by multiplex PCR (m-PCR)

All the *E. coli* isolates including controls were subjected to m-PCR for detection of stx_1, stx_2, *eae*, *ehxA* and *saa* genes considered for virulence factor of Shiga toxin–producing *E. coli* (STEC) as described by Paton and Paton (1998b, 2002). The amplified product was visualized by gel documentation system (UVP) after electrophoresis in 2% (W/V) agarose gel containing ethidium bromide (0·5 μg ml^{-1}) (SRL, India) (Sambrook and Russel 2001).

Subtyping of stx_1 gene present in STEC isolates from buffalo

All the stx_1-positive STEC isolates including the controls were subjected to PCR amplification for the presence of stx_{1c} gene as described earlier (Zhang *et al.*2002).

Subtyping of stx_2 gene present in STEC isolates from buffalo by Multiplex PCR

All the stx_2-positive STEC isolates including the controls were also subjected to m-PCR amplification for the presence of stx_{2o}, stx_{2d}, stx_{2e} and stx_{2f} genes as previously described (Wang *et al.* 2002).

Molecular characterization of STEC strains by randomly amplified polymorphic DNA-PCR (RAPD-PCR)

The molecular characterization of all the STEC isolates was performed by RAPD-PCR using a single primer 1247 (Merck Biosciences) in Gene Amp PCR system 9700 (Applied Biosystems, USA) as per the protocol of Khan *et al.*(2002a). The PCR products were then electrophoresed in 1% (W/V) agarose gel containing ethidium bromide (0·5 μg ml^{-1}) (Sambrook and Russel 2001).

Detection of phylogenetic relationship among STEC isolates

All the images taken by the gel documentation system (UVP) were analysed using the Doc-itLs image analysis software supplied with the system (UVP) as per manufacturer's instruction. By comparing the difference in the RAPD-PCR banding pattern, phylogenetic relationship between the isolates was established. An unrooted phylogenetic tree was made using neighbour-joining method, available in the software.

Screening for *katp, etpD* and *toxB* in STEC isolates by PCR

The PCR for the detection of *katp* and *etpD* genes was performed using Gene Amp PCR system 9700 (Applied Biosystems, USA) as per the procedure described by Khan *et al.* (2002b). The presence of *toxB* gene was detected by PCR as per the method described by Tarr *et al.* (2002).

Antibiotic Sensitivity test of Shiga toxin–producing *Escherichia coli* (STEC) isolates

All the STEC isolates were tested for their sensitivity and resistance to different antibiotics by the disc diffusion method (CLSI 2008). The antibiotics used were chloramphenicol (25 μg), co-trimoxazole (25 μg), ciprofloxacin (5 μg), gentamicin (10 μg), neomycin (30 μg), norfloxacin (10 μg), streptomycin (30 μg), oxytetracycline (30 μg), cephalothin (30 μg), amikacin (30 μg), ceftazidime (30 μg), kanamycin (30 μg), ceftriaxone (30 μg), levofloxacin (5 μg), amoxicillin + clavulanic acid (20/10 μg), cefaclor (30 μg), cefuroxime (30 μg), azithromycin (30 μg), piperacillin + tazobactam (100 + 10 μg), cefepime + tazobactam (30 + 10 μg), ampicillin + cloxacillin (10 μg), enrofloxacin (10 μg), amoxicillin (25 μg), erythromycin (10 μg), doxycycline hydrochloride (30 μg) and pefloxacin (5 μg) (Hi Media).

Detection of ESBL-producing and quinolone resistance genes in STEC isolates

All the Shiga toxin–producing *E. coli* isolates including controls were subjected to PCR for the detection of $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV} and *qnrA* genes using the primers and the cycle conditions as described earlier (Weill *et al.* 2004a,b; Castanheira *et al.* 2007). The primers were procured from M/s Genetix Biotechnology Asia Private Limited. The amplified product was visualized by gel documentation system (UVP) after electrophoresis in 2% (W/V) agarose gel containing ethidium bromide (0·5 μg ml^{-1}) (SRL) (Sambrook and Russel 2001).

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