Molecular characterization and antibiotic susceptibility pattern of caprine Shiga toxin producing-*Escherichia coli* (STEC) isolates from India

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Summary

The present study was conducted to detect the occurrence, serotype, genotype, phylogenetic relationship and antimicrobial resistance pattern of STEC from healthy goats of West Bengal, India. From the 125 faecal samples collected from healthy goats, 245 isolates were identified as *Escherichia coli*. The *E. coli* harbouring any gene for Shiga toxins (stx_1/stx_2) was detected in 36 (14.7%) of the 245 *E. coli* isolates. These STEC strains belonged to 22 different serogroups (O2, O5, O20, O21, O22, O25, O41, O44, O45, O60, O71, O76, O84, O85, O87, O91, O103, O112, O113, O120, O156, and O158) and three were untypeable. The stx_1 and stx_2 was detected in 26 (72.2%) and 21 (58.3%) of Shiga toxin producing-*E. coli* (STEC) isolates, respectively. Further, *E. coli* harbouring *eaeA* only (Enteropathogenic *E. coli*) and *ehxA* was detected in 22 (61.1%) and 28 (77.7%) isolates, respectively. Whereas the *saa* was present in 8 (22.2%) *E. coli* isolates. The subtyping of the 26 *E. coli* strains possessing stx_1 showed that 73.% (19/26) of these isolates were positive for stx_{1C} subtype. Of the 21 isolates with the stx_2 gene, 42.8% (9/21) were positive for stx_{2C}, and 38.1% (8/21) were positive for stx_{2d} gene. The phylogenetic analysis of STEC strains after RAPD reveals eight major clusters. However, no serogroup specific cluster was observed. Resistance was observed most frequently to erythromycin (80.5%), amikacin (52.7%), cephalothin (50%), kanamycin (41.6%), neomycin (36.1%) and gentamycin (36.1%) and less frequently to norfloxacin (2.7%), enrofloxacin (2.7%), and ciprofloxacin (2.7%). Multidrug resistance was observed in eleven STEC isolates.

Key words: Goat, EPEC, STEC, West Bengal

Introduction

Shiga toxin producing-*Escherichia coli* (STEC) are food borne pathogens associated with a spectrum of human illness ranging from bloody diarrhoea to life-threatening infection such as hemolytic uraemic syndrome (HUS), thrombotic thrombocytopenic purpurea (TTP) and hemorrhagic colitis (HC). The bacteria produce the life-threatening infection with the help of Shiga toxins, encoded by stx_1 and stx_2 genes and the products of the locus of eneterocyte effacement (LEE) pathogenicity island such as *eaeA* gene. The *eaeA* gene encodes for the "intimin" protein which is involved in the intimate adhesion of bacteria to the enterocytes and production of attaching and effacing (AE) lesion (Paton and Paton, 1998a). Some LEE negative strains can also possess *saa* gene encoding a novel outer membrane protein which acts as auto agglutinating adhesin (Paton *et al*., 2001). The majority of STEC strains also have hemolytic phenotype in washed sheep blood agar due to production of enterohemolysin, encoded by *ehxA* gene (Beutin *et al*., 1989).

The domestic and wild ruminants act as the major reservoir of STEC which can secrete the bacteria through the faeces (Wani *et al*., 2004). Due to absence of clinical manifestation the reservoir animals are included in the food production. The food products from the reservoir animals are at risk of contamination with STEC either with their faeces or intestinal contents during slaughter (Asakura *et al*., 2014). Thus, STEC can enter the human food chain through contaminated food products and is responsible for several food borne outbreaks as reported earlier (CDC, 2008; Ethelberg *et al*., 2009). So, the present study was conducted to detect the occurrence, serotype, genotype, phylogenetic relationship and antimicrobial resistance pattern of STEC from healthy goats of West Bengal, India. Multiplex PCR was used for primary screening of *E. coli* isolates for Shiga toxin genes, *eaeA* and *ehxA*. PCR based subtyping of the Shiga toxin genes, serogrouping and RAPD based phylogenetic relationship were determined among the isolates.

Materials and Methods

Sampling

A total of 125 faecal samples were collected from

healthy goats in different locations of West Bengal, India. The samples were transported to the laboratory in transport medium in sterile vials on ice for further processing. None of the samples were held for more than 24 h at 4°C.

Isolation of *Escherichia coli*

The collected samples were inoculated into the nutrient broth and were incubated at 37°C for 24 h (HiMedia, India). The growth in the nutrient broth was transferred to MacConkey's agar (HiMedia, India) and again incubated at 37°C overnight. The next day 2-3 rose pink colonies were randomly picked and transferred to EMB agar (HiMedia, India) followed by an overnight incubation at 37°C. The colonies were observed for metallic sheen and single colony was streaked into nutrient agar 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 earlier (Quinn *et al*., 1994). The strains were preserved in glycerol broth at -70°C for subsequent characterization.

Multiplex-PCR for detection of *stx*$_1$, *stx*$_2$, *eaeA*, *ehxA*, *saa* gene sequences

For PCR based detection of STEC genes, all the *E. coli* isolates were subjected to DNA extraction as per previously described method of Samanta *et al*. (2014). All the *E. coli* isolates were subjected to multiplex-PCR (m-PCR) for the detection of Shiga toxin gene(s) (*stx*$_1$, *stx*$_2$), intimin (*eaeA*), enterohaemolysin (*ehxA*) and STEC autoagglutinating adhesin (*saa)* as per the previously described primers and protocol (Paton and Paton, 1998b, 2002; Paton *et al*., 2001). The m-PCR was performed in a gradient thermocycler (Eppendorf ProS, Germany). All the reagents and oligo nucleotide primers were procured from Genetix Biotechnology Asia Private Limited, India. The amplified DNA products were visualized by Gel Documentation System (UVP, UK) after gel electrophoresis in 2% agarose containing ethidium bromide (0.5 µg ml^{-1}) (SRL, India) as per the standard protocol (Sambrook and Russel, 2001).

Subtyping of *stx*$_1$ and *stx*$_2$ gene

All the *stx*$_1$ positive *E. coli* isolates were subjected to PCR amplification for the presence of *stx*$_{1c}$ gene by using the previously described method of Zhang *et al*. (2002). Further, all the *stx*$_2$ positive *E. coli* isolates were subjected to m-PCR amplification for the presence of *stx*$_{2c}$, *stx*$_{2d}$, as described (Wang *et al*., 2002).

Serogrouping

The *E. coli* isolates harbouring characteristic virulence genotype were O-serogrouped from National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kausali, Himachal Pradesh, India.

Molecular characterization of STEC isolates and phylogenetic analysis

All the STEC isolates were subjected to RAPD-PCR as described (Mahanti *et al*., 2013). The amplified DNA products were visualized by Gel Documentation System (UVP, UK) after gel electrophoresis in 1% agarose containing ethidium bromide (0.5 µg ml^{-1}) (SRL, India) as per the standard protocol (Sambrook and Russel, 2001). An unrooted phylogenetic tree was created by using neighbour joining method in the Doc-itLs image analysis software (UVP, UK).

Antibiotic sensitivity test of 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 (30 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).

Results

Prevalence of Shiga toxin producing-*E. coli*

From the 125 faecal samples collected from healthy goats of different places in West Bengal, India, 245 isolates were identified as *E. coli* after morphological and biochemical confirmation. The *E. coli* harbouring any gene for Shiga toxins (*stx*$_1$/*stx*$_2$) was detected in 36 (14.7%) of the 245 *E. coli* isolates. These STEC strains belonged to 22 different serogroups (O2, O5, O20, O21, O22, O25, O41, O44, O45, O60, O71, O76, O84, O85, O87, O91, O103, O112, O113, O120, O156, and O158) and three were untypeable (Table 1).

The *stx*$_1$ and *stx*$_2$ were detected in 26 (72.2%) and 21 (58.3%) of STEC isolates, respectively. Further, *E. coli* harbouring *eaeA* only (Enteropathogenic *E. coli*) and *ehxA* was detected in 22 (61.1%) and 28 (77.7%) isolates, respectively. Whereas the *saa* was present in 8 (22.2%) *E. coli* isolates from goat in this study, out of which 5 (62.5%) were associated with the *ehxA* gene (Table 1, Fig. 1).

Subtyping of *stx*$_1$ and *stx*$_2$

The subtyping of the 26 *E. coli* strains possessing *stx*$_1$ showed that 73.% (19/26) of these isolates were positive for *stx*$_{1C}$ subtype. Most of the *stx*$_{1C}$ possessing strains occurred in combination with *ehxA*, *saa* or both. Of the 21 isolates with the *stx*$_2$ gene, 42.8% (9/21) were positive for *stx*$_{2C}$, and 38.1% (8/21) were positive for *stx*$_{2d}$ gene (Table 1, Fig. 1).

Molecular characterization of STEC isolates and phylogenetic analysis

All 36 STEC strains were characterized by RAPD-

Table 1: Serogroup and genotype of the STEC and EPEC isolates from goats in West Bengal, India

Serial No.	Serogroup	Genetype
G1	O5	*stx$_{2C}$*, *eae*, *ehxA*
G2	O45	*stx$_1$*, *eae*, *ehxA*
G3	O84	*eae*, *ehxA*
G4	O76	*stx$_1$*, *stx$_2$*, *ehxA*, *saa*
G5	O91	*stx$_{1C}$*, *stx$_{2d}$*, *ehxA*
G6	O76	*eae*, *ehxA*
G7	O25	*stx$_{1C}$*, *stx$_{2d}$*, *eae*, *ehxA*
G8	O112	*stx$_{1C}$*, *stx$_{2d}$*, *saa*
G9	O91	*stx$_{2C}$*, *eae*, *ehxA*
G10	O6O	*stx$_{1C}$*, *eae*, *ehxA*
G11	O120	*stx$_{1C}$*, *stx$_{2d}$*, *ehxA*, *saa*
G12	O5	*stx$_{1C}$*, *eae*, *ehxA*
G13	O44	*stx$_{1C}$*, *eae*, *ehxA*
G14	O113	*stx$_1$*, *eae*, *ehxA*
G15	O21	*stx$_{2d}$*
G16	O22	*stx$_1$*, *eae*, *ehxA*
G17	OUT	*stx$_{1C}$*, *stx$_2$*, *eae*, *ehxA*
G18	O156	*stx$_1$*, *stx$_{2d}$*
G19	O87	*stx$_{1C}$*, *stx$_{2C}$*, *ehxA*
G20	O2	*stx$_{1C}$*, *stx$_2$*, *ehxA*, *saa*
G21	O85	*stx$_1$*, *eae*
G22	O60	*stx$_{1C}$*, *stx$_2$*, *ehxA*, *saa*
G23	O87	*stx$_{1C}$*, *eae*, *ehxA*
G24	OUT	*eae*, *ehxA*
G25	O158	*stx$_{2d}$*, *saa*
G26	O5	*stx$_{2C}$*, *eae*, *ehxA*
G27	O41	*stx$_{1C}$*, *stx$_{2C}$*
G28	O113	*stx$_{1C}$*, *stx$_{2C}$*, *ehxA*, *saa*
G29	OUT	*stx$_{1C}$*, *eae*, *ehxA*
G30	O103	*stx$_{1C}$*, *eae*, *ehxA*
G31	O120	*stx$_{1C}$*, *eae*, *ehxA*
G32	O112	*stx$_{1C}$*, *eae*, *ehxA*
G33	O22	*stx$_{2d}$*
G34	O71	*stx$_{2C}$*, *eae*, *ehxA*
G35	O20	*stx$_{1C}$*, *stx$_{2C}$*, *saa*
G36	O5	*stx$_1$*, *stx$_{2C}$*, *eae*, *ehxA*

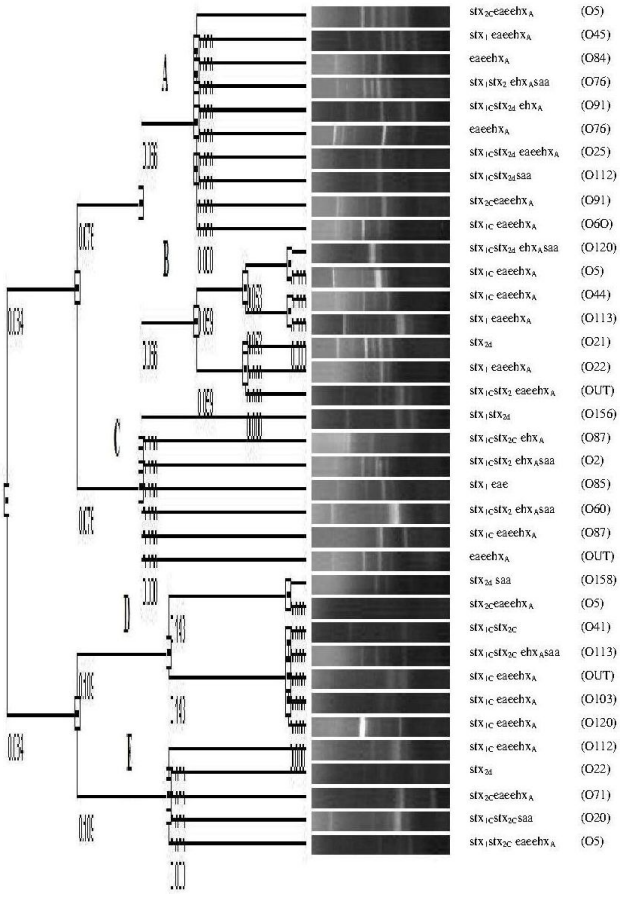

Fig. 1: Phylogenetic analysis using RAPD-PCR profiles of the STEC and EPEC strains isolated from goats in West Bengal (India) using neighbour-joining method and dice similarity

Table 2: Antibiotic resistance pattern of the STEC and EPEC from goats in West Bengal, India

Name of the antimicrobial agent (Conc. µg)	Sensitive (%)	Intermediate (%)	Resistant (%)
Chloramphenicol (25 µg)	77.78	11.11	11.11
Co-trimoxazole (25 µg)	77.78	16.67	5.55
Ciprofloxacin (5 µg)	88.89	8.33	2.78
Gentamicin (10 µg)	5.56	58.33	36.11
Neomycin (30 µg)	0	63.89	36.11
Norfloxacin (10 µg)	88.89	8.33	2.78
Streptomycin (30 µg)	5.55	77.78	16.67
Oxytetracycline (30 µg)	19.44	55.56	25
Cephalothin (30 µg)	5.56	44.44	50
Amikacin (30 µg)	2.78	44.44	52.78
Ceftazidime (30 µg)	97.22	2.78	0
Kanamycin (30 µg)	5.55	52.78	41.67
Ceftriaxone (30 µg)	88.89	11.11	0
Levofloxacin (5 µg)	100	0	0
Amoxicillin + clavulanic acid (30 µg)	80.56	13.89	5.55
Cefaclor (30 µg)	36.11	38.89	25
Cefuroxime (30 µg)	72.23	19.44	8.33
Azithromycin (30 µg)	11.11	69.45	19.44
Piperacillin + tazobactam (100 + 10 µg)	52.78	47.22	0
Cefepime + tazobactam (30 + 10 µg)	100	0	0
Ampicillin + cloxacillin (10 µg)	69.45	22.22	8.33
Enrofloxacin (10 µg)	80.55	16.67	2.78
Amoxicillin (25 µg)	75	19.45	5.55
Erythromycin (10 µg)	0	19.45	80.55
Doxycycline hydrochloride (30 µg)	36.11	55.56	8.33
Pefloxacin (5 µg)	75	19.45	5.55

PCR to determine the genetic diversity among the strains. All the strains were typeable with primer 1247 and produced amplified fragment size ranging from 250 bp to 1900 bp (calculated by Doc-itLs image analysis software, UVP, UK). The phylogenetic analysis of STEC strains after RAPD reveals eight major clusters (clusters

A to E, Fig. 1). No serogroup specific cluster was observed.

Antibiotic sensitivity test

Resistance was observed most frequently to erythromycin (80.5%), amikacin (52.7%), cephalothin (50%), kanamycin (41.6%), neomycin (36.1%) and gentamycin (36.1%) and less frequently to norfloxacin (2.7%), enrofloxacin (2.7%), and ciprofloxacin (2.7%). No resistance was observed in case of ceftazidine, levofloxacin, piperacillin along with tazobactam, cefepime along with tazobactam and ceftriaxone. Multidrug resistance was observed in eleven STEC isolates (Table 2).

Discussion

The study was aimed to detect the occurrence of STEC from healthy goats in West Bengal, India. Of the total 245 *E. coli* isolated during the present study 36 (14.7%) isolates harboured at least one marker gene for STEC. Similar occurrence of STEC was observed in previous studies conducted in local and migratory goats in Kashmir Valley, India (Wani *et al*., 2006) and from faecal samples, meat and milk of goats in other parts of the world (Orden *et al*., 2003; Islam *et al*., 2008; Momtaz *et al*., 2013). However, lower or higher occurrence of STEC was detected from faeces of healthy and diarrhoeic goats, chevon and goat milk cheese in other countries throughout the world (Orden *et al*., 2003; Horcajo *et al*., 2010; Ojo *et al*., 2010; Schilling *et al*., 2012). It seems that the occurrence of STEC varies with different geographical location and season.

All the STEC strains belonged to 22 different serogroups (O2, O5, O20, O21, O22, O25, O41, O44, O45, O60, O71, O76, O84, O85, O87, O91, O103, O112, O113, O120, O156, and O158) and three were untypeable. Similarly, the serogroups O2, O5, O22, O45, O60, O76, O87, O91, O103, O112, O113 and O156 were also previously isolated from faeces and milk product of goats (Wani *et al*., 2006; Vu-Khac and Cornick, 2008; Horcajo *et al*., 2010; Schilling *et al*., 2012; Jacob *et al*., 2013).

Out of the 36 STEC isolates of the present study, 26 (72.2%) and 21 (58.3%) isolates possessed the stx_1 and stx_2 gene, respectively. Similar occurrence of stx_1 and higher occurrence of stx_2 was observed in goats in previous studies (Oliveira *et al*., 2008; Vu-Khac and Cornick, 2008). Further characterization of the 26 stx_1 positive isolates identified 19 (73%) strains positive for stx_{1C}. Similarly, higher number of stx_{1C} was observed in stx_1 positive isolates from goats in Spain (Horcajo *et al*., 2010) and Central Vietnam (Vu-Khac and Cornick, 2008). In this study, we found that stx_{1C} genes most commonly occurred with *ehxA*, *saa* or both which is in agreement with the previous finding by Vu-Khac and Cornick (2008). Subtyping of the isolates possessing stx_2 gene, produced 9 (42.8%) and 8 (38.1%) isolates positive for stx_{2C} and stx_{2d} gene, respectively. Similarly, Zheng *et al*. (2005) found high proportion of isolates bearing stx_{2C} genes in healthy goats in China which is consistent with the present finding. In contrast, higher recovery of stx_{2d} gene has been observed in caprine isolates in Central Vietnam (Vu-Khac and Cornick, 2008). It seems that the prevalence of stx_{2C} isolates is higher in this geographical region as China is nearer than Vietnam to the present study area.

The *eaeA* gene coding intimin protein was present in 22 (61.1%) isolates. Lower occurrence of *eaeA* was found in a study conducted by Vu-Khac and Cornick (2008), whereas Islam *et al*. (2008) could not isolate *eaeA* in non-O157 STEC from goats. Higher prevalence of *eaeA* in the present study may be a concern as LEE positive strains were considered highly virulent in human (Toma *et al*., 2004). A large number of isolates (77.7%) was found to possess enterohaemolysin (*ehxA*) in combination with other virulence genes in the present study which is in contrast with the previous study (Vu-Khac and Cornick, 2008). Association of *saa* gene in STEC isolates (22.2%) from healthy goats in the present study might be remarkable because in another study, *saa* was not detected in caprine STEC isolates (Cortes *et al*., 2005). Among the *saa* positive isolates 5 (62.5%) were concomitantly present with the *ehxA*, which is consistent with the previous studies (Paton *et al*., 2001; Paton and Paton, 2002).

The RAPD results showed genetic heterogenicity of the STEC isolates. No serogroupwise clustering of the STEC isolates in the present study was detected. This is in agreement with the previous observation by Mahanti *et al*. (2013) who found many RAPD banding patterns among the STEC isolates from buffalo belonging to a similar serogroup.

Resistance of STEC isolates against chloramphenicol, co-trimoxazole, ciprofloxacin, gentamicin, neomycin, norfloxacin, streptomycin, tetracycline, ampicillin, enrofloxacin, erythromycin as reported in the present study was also supported by earlier studies in goats (Ojo *et al*., 2010; Medina *et al*., 2011). However, higher occurrence of multidrug resistant STEC (69.5%) was reported in Nigerian goats than in the present study (Ojo *et al*., 2010). Isolation of enterovirulent *E. coli* such as STEC and EPEC is a serious public health concern in this area where the farmers stay in close proximity to goats with little concern for hygiene and people consume undercooked goat milk and meat products.

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