Prevalence of Extended-spectrum and AmpC Beta-lactamases Producing STEC in Bovine Diarrhoea Cases in West Bengal, India

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A B S T R A C T

Escherichia coli are important commensal with pathogenic potentiality in bovine neonates and may cause intestinal and extra-intestinal infections. Different enteric pathogens such as *E. coli* may remain associated with bovine diarrhoea which is a multifactorial menace. Possession of antimicrobial resistance genes by these commensal or pathogens associated with diarrhea makes the issue more serious. The study was aimed to detect the occurrence and characterize extended-spectrum (ESBL) and AmpC (ACBL) beta-lactamases producing pathogenic *E. coli* (STEC) in diarrhoeal faecal samples collected from bovines. All the *E. coli* strains (13/21, 61.9%) isolated from bovine diarrhoea cases (n=21) were found to be ESBL producers. In total, 11 (84.6%) isolates were positive in cefoxitin–cloxacillin double-disc synergy (CC-DDS) test for AmpC production and the isolates were also confirmed by PCR for bla_{AmpC}. Any of the *E. coli* isolates were not carrying the class I integrons but 7 (53.8%) of those isolates were found to possess the STEC gene, *eaeA* (responsible for causing diarrhoea) as revealed in PCR assays. All the ESBL producing *E. coli* isolates showed a high level of resistance to amoxicillin/clavulanic acid, ceftriaxone, ceftazidime, ampicillin/cloxacillin, cefotaxime, cefepime, cefoperazone (100%), cefoxitin (84.6%) and tetracycline (61.5%) whereas Ertapenem (92.3%), and enrofloxacin (84.6%) were intermediately sensitive against these pathogens. All the isolates were susceptible to amikacin, levofloxacin, gentamicin, ampicillin/sulbactam, and doxycycline.

Introduction

Neonatal Calf Diarrhoea/bovine diarrhoea is a multifactorial syndrome including pathogen (infectious NCD) as well as non-infectious factors related to the host (immunological and nutritional status), the environment and the management (Izzo *et al.,* 2011). Because of the multifactorial nature of NCD, *E. coli* is one of the causes, is difficult to control effectively (Cho and Yoon, 2014).

Enterotoxigenic *E. coli* (ETEC) are considered as the most common *E. coli* pathotype associated with NCD and the strains produce K99 (F5) adhesin and heat-stable (STa or STb) and/or heat-labile (LT1 or LT2) enterotoxins (Kaper *et al.,* 2004). The

ETEC causes watery diarrhoea and weakness in 1–4days old newborn calves. Death usually occurs within 24 hours of infection due to severe dehydration (Cho *et al.,* 2010). The fimbrial adhesin F5 (K99) promotes the adhesion of bacterial cells to glycoproteins on the epithelial surface of the jejunum and/or ileum, and bacterial enterotoxins cause damage to the epithelial cells, resulting in fluid secretion and subsequently the diarrhoea (Acres, 1985). Shiga toxin-producing *Escherichia coli* (STEC) and Enterohemorrhagic *Escherichia coli* (EHEC) are also dreadful animal pathogens causing the infection through food and water leading to gastrointestinal infections and bloody diarrhoea in bovines (Hickey *et al.,* 2015; Nguyen and Sperandio, 2012).

Many STEC also produces intimin, an outer membrane surface adhesin encoded by the chromosomal *eaeA* gene with several variants found in the pathogenicity island (locus of enterocyte effacement [LEE]) responsible for causing bloody diarrhoea in calves (Blanco *et al.,* 2004). Enterotoxigenic *Escherichia coli* (ETEC) can cause bovine infections leading to diarrhoea with morbidity and mortality loss throughout the world (Izzo *et al.,* 2011; Acha *et al.,* 2004).

The possession of antimicrobial resistance genes like the genes encoding ESBL and ACBL in *E. coli* strains is the growing concern in the globe including India. The pathogens carrying the resistance genes pose a major challenge for the treatment of infections as the enzymes encoded make the pathogen resistant to higher generation of cephalosporins (Tenover *et al.,* 1999). Consequently, there is an increase in the use of last-resort antimicrobial drugs (*i.e.* carbapenems) for treatment. Again, *E. coli* strains carrying the resistance genes can easily transfer those genes to other pathogens leading to the spread of the determinants (Hu

et al., 2016). In this background, the present study was aimed for the detection and characterization of ESBL and ACBL producing pathogenic *E. coli* strains (STEC) from diarrhoeic faecal samples of calves in West Bengal (India) followed by further characterization and to know their antibiotic resistance patterns *in-vitro*.

Materials and Methods

Collection & transportation of faecal samples

Twenty-one faecal samples (n=21) from bovine diarrhoea cases were aseptically collected from few private owners from Nadia district of West Bengal during April 2019. The faecal samples were collected directly into sterile vials with the sterile swabs from the rectum of the animals.

All the collected swabs were kept into the sterile peptone water (HiMedia, India) for transport. All the samples collected were placed on ice in a thermos flask with the proper label and were brought to the laboratory for further processing within 48hrs of collection.

Isolation of Escherichia coli from faecal samples

The peptone water (HiMedia, India) containing faecal samples were incubated at 37^0C for overnight. After that, it was streaked onto MacConkey's agar (HiMedia, India) and again incubated at 37^0C for overnight. Next day 2-3 rose pink colonies were randomly picked and transferred to EMB agar (HiMedia, India). The plates were incubated overnight at 37^0C. Next day colonies were observed and a single colony was streaked to nutrient agar (HiMedia, India) slant for further biochemical confirmation.

Morphological and Biochemical identification

All the pure cultures were subjected to different morphological and biochemical tests as per methods described by Quinn *et al.,* (2011).

Detection of Escherichia coli by PCR

Bacterial DNA extraction

For genotypic detection *Escherichia coli,* DNA was extracted from all the *Escherichia coli* isolates as per Mahanti *et al.,* (2013).

Detection of Escherichia coli in the faecal sample by PCR

The morphologically and biochemically confirmed *Escherichia coli* isolates were subjected to PCR for genotype-based detection of the 16S *rRNA* gene as described by Wang *et al.*(1996) with some modification.

Detection of ESBL Property in Escherichia coli isolates

Phenotypic Confirmatory Tests for ESBL Production

The antibiotic discs containing cefotaxime (30μg, HiMedia) and ceftazidime (30μg, HiMedia) with or without clavulanate (10μg, HiMedia) were used and a difference of ≥5 mm between the zone diameters of either of the cephalosporin disks and their respective cephalosporin/clavulanate disk was considered to be phenotypically positive for ESBL propertyin the isolates(Bauer *et al.,* 1966; Patel *et al.,* 2015).

Detection of bla_{CTX-M}, $bla_{TEM}$and bla_{SHV} Genes in Escherichia coli isolates by PCR

Detection of bla_{CTX-M}, bla_{TEM} and bla_{SHV} genesin phenotypically ESBL producing *Escherichia coli* isolate by PCR assay was performed as per the protocol of Weill *et al.,* (2004) and Cao *et al.,* (2002) with some modification, respectively.

Detection of ACBL Production in Escherichia coli isolates

Phenotypic assays for detection of AmpC beta-lactamase in Escherichia coli isolates

All the *Escherichia coli* including ESBL producing isolates were subjected to cefoxitin– cloxacillin double-disc synergy (CC-DDS) test for phenotypic confirmation of ACBL property following the previously described protocol (Tan *et al.,* 2009).

Detection of AmpC Gene in Escherichia coli isolates by PCR

Detection of bla_{AmpC} genein phenotypically positive ACBL producing *Escherichia coli* isolates by PCR assay was performed as per the protocol of Féria*et al.,* (2002) with some modification.

Phenotypic assays for detection of carbapenemase and metallobeta-lactamase production in Escherichia coli isolates

All the Ertapenem intermediate ESBL producers were subjected to modified Hodge test (MHT) and combination disc diffusion test (CDDT) [IMP-10 µg and IMP-10 µg + EDTA-750µg] to confirm carbapenemase and Metallo beta-lactamase (MBL) production**,** respectively (Birgy *et al.,* 2012).

Detection of Class I integrons in ESBL and ACBL producing Escherichia coli isolates

All the ESBL and ACBL producing *Escherichia coli* isolates from faecal samples were studied for the presence of class I integrons as per the previously described

methods of Mazel *et al.,* (2000) and Buffet-Bataillon *et al.,* (2012) and with some modifications.

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

All *E.coli* isolates including positive control were subjected to multiplex PCR for detection of any of the *stx_1, stx_2, eaeA* and *ehxA* genes considered for virulence factor of Shiga-toxin producing *E.coli* (STEC) as per the previously described protocol of Paton and Paton (1998, 2002).

Antimicrobial Sensitivity test of the ESBL producing E. coli isolates

Allthe ESBL producing isolates were tested for their sensitivity and resistance to different antimicrobials by the disc diffusion method (Bauer *et al.,* 1966, Patel *et al.,* 2015). The antibiotic discs used were Ertapenem (10μg), enrofloxacin (5μg), cefoperazone (75 μg), cefoxitin (30μg), cefepime (30μg), gentamicin (10μg), tetracycline (30μg), amikacin (30μg), levofloxacin (5μg), ceftriaxone (30μg), ampicillin/sulbactam (10/10μg), ceftazidime (30μg), cefotaxime (30μg), ampicillin + cloxacillin (10μg), doxycycline hydrochloride (30μg) and amoxyclav (30μg).

Molecular characterization of isolated ESBL producing E. coli strains by Randomly amplified polymorphic DNA-PCR (RAPD-PCR)

The molecular typing of all the ESBL producing isolates was done by RAPD-PCR using a primer (GTG)$_5$ (GCC Biotech, India) in Gene Amp PCR system 9700 (Applied Biosystems, USA) as per previously described methods by described by Kar *et al.,* (2015) with some modifications.

Interpretation of the RAPD-PCR results

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

Results and Discussion

Colibacillosis is an economically important disease of bovines. This study was aimed to assess the risk factors associated with *E. coli* among diarrhoeic cattle.In the present study, 13 (61.9%) bovine diarrhoeal faecal samples showed growth of the characteristic pink-coloured colony in MacConkey agar and the isolates also produced 'metallic sheen' on EMB agar plates and all these isolates were presumptively considered as *E. coli*. Further characterization showed these were Gram-negative small rods and showed standard results of biochemical tests like Catalase (+ve), Oxidase (-ve), Urease (-ve), and Indole-Methyl Red-VogesProskauer-Citrate (+ + - -) [Quinn *et al.,* 2011; Samanta, 2013]. All 13*E. coli*isolates were confirmed to be *Escherichia coli* in the 16S *rRNA* gene PCRand this report is quite similar to the findings of Paul *et al.*(2010) who isolated 76% *E. coli* positivity from calf faecal samples. Other studies conducted by Masud *et al.,* (2012), Dereje (2012), Taghadosi *et al.,* (2018) and Mohammed *et al.,* (2019) also detected 20(44%), 25(43.1%), 41(26.3%) and 26 (46.4%) *E. coli* isolates, respectively from bovine diarrhoeic faecal samples which showed a lower prevalence of *E. coli* than the present findings. Consideration of 2-3 rose

pink colonies per sample in the present study is the probable reason for the higher occurrence.

During the detection of ESBL production phenotypically, 100% *E. coli* isolates were found to be positive for ESBL production (in combined disc diffusion assay) followed by confirmation of having at least one of the common ESBL genes (Klimiene *et al.,* 2018). All the isolates harboured the bla_{CTX-M}(100%) whereas11(84.6%) isolates possess bla_{SHV} gene, however, none of the isolates possessed the bla_{TEM} gene (Table 1). This study revealed that bla_{CTX-M} was the most prevalent gene followed by bla_{SHV}. The findings are corroborative with the reports of Geser *et al.,* (2012) [bla_{CTX-M} - 94%, bla_{SHV} - 6%, bla_{TEM}- 0%], Kar *et al.,* (2015) [bla_{CTX-M} - 72%, bla_{SHV} - 94%, bla_{TEM} - 50%] and Upadhyay*et al.,* (2015)[bla_{CTX-M} - 56.3%, bla_{SHV} - 9.7%, bla_{TEM} - 12.6%] who also reported CTX-M as

the dominant ESBL genotype in *E. coli* isolates from different sources. ESBL positivity in *E. coli* isolates was also reported from different countries by Casella *et al.,* (2017) and Maamar *et al.,* (2016).No detection of bla_{TEM} gene in ESBL *E. coli* isolates was also supported by Geser *et al.,* (2012).

The double-disc synergy using cefoxitin–cloxacillin (CC-DDS) test for phenotypic confirmation of ACBL production showed 11(84.6%) isolates were AmpC producers and were confirmed to possess bla_{AmpC} gene in PCR assay (Table 1). This high-level detection ACBL property in *E. coli* is almost matching with the reports of Vinueza-Burgos *et al.,* (2019) [94.3%], Banerjee and Acharyya (2020) [88.9%] but quite higher than the reports of Kar *et al.,* (2015) [11.1%] and Casella *et al.,* (2017) [4.2%] maybe possibly due to geographical variation.

Table.1 Genotyping of ESBL and ACBL producing & STEC *E. coli* strains isolated from bovine diarrhoeal faecal samples in West Bengal, India

Sl. No.	Detection of STEC gene (*eaeA*)	Phenotypic Detection of			Molecular Detection of			
		ESBL property		ACBL property	ESBL Genes			ACBL Gene
		DDCTX	DDCAZ	DDCX	bla_{CTXM}	bla_{TEM}	bla_{SHV}	bla_{AmpC}
1	-	+	+	-	+	-	+	-
2	+	+	+	+	+	-	+	+
3	+	+	+	+	+	-	-	+
4	-	+	+	+	+	-	+	+
5	-	+	+	-	+	-	+	-
6	+	+	+	+	+	-	+	+
7	-	+	+	+	+	-	+	+
8	+	+	+	+	+	-	-	+
9	-	+	+	+	+	-	+	+
10	-	+	+	+	+	-	+	+
11	+	+	+	+	+	-	+	+
12	+	+	+	+	+	-	+	+
13	+	+	+	+	+	-	+	+

Table.2 Antibiogram of ESBL producing pathogenic *E. coli* strains isolated from bovine diarrhoea cases in West Bengal, India

Sl. No.	Antimicrobials (Conc. in µg)	Isolates sensitive		Isolates intermediately sensitive		Isolates resistant	
		No.	%	No.	%	No.	%
1.	Amikacin (AK - 30)	13	100	0	0	0	0
2.	Amoxicillin/Clavulanic acid (AMC - 20/10)	0	0	0	0	13	100
3.	Ceftriaxone (CTR - 30)	0	0	0	0	13	100
4.	Ampicillin/Sulbactam (A/S-10/10)	12	92.31	1	7.69	0	0
5.	Doxycycline (DO - 10)	12	92.31	1	7.69	0	0
6.	Ceftazidime (CAZ - 30)	0	0	0	0	13	100
7.	Ertapenem (ETP - 10)	1	7.69	12	92.31	0	0
8.	Gentamicin (GEN - 10)	13	100	0	0	0	0
9.	Levofloxacin (LE - 5)	13	100	0	0	0	0
10.	Ampicillin/Cloxacillin (AX-10)	0	0	0	0	13	100
11.	Cefoxitin (CX - 30)	0	0	2	15.38	11	84.62
12.	Cefotaxime (CTX - 30)	0	0	0	0	13	100
13.	Tetracycline (TE - 10)	5	38.46	0	0	8	61.54
14.	Enrofloxacin (EX - 5)	0	0	11	84.62	2	15.38
15.	Cefepime (CPM - 30)	0	0	0	0	13	100
16.	Cefoperazone (CPZ - 75)	0	0	0	0	13	100

None of the Ertapenem intermediate isolates was positive for the production of carbapenemase and metallobeta-lactamase enzyme and was carrying the class I integrons as revealed in this study. This might be due to no use of carbapenems in the therapy of bovine diarrhoea or other clinical conditions in this study area. Transmission of ESBL/ACBL producing organisms is regulated by mobile genetic elements especially class 1 integrons. Sometimes class 1 integrons detected from animals and human ACBL/ESBL producing isolates are homologous which confirms their role in transmission (EFSA Panel on Biological Hazards, 2011). Absence of integrons in the present study indicated the low transmission possibilities of the isolates. Similarly, the absence of class 2 and 3 integrons was detected earlier in drug-resistant *E. coli* isolates (Huang *et al.,* 2020).

Multiplex PCR assays (m-PCR) revealed that 7 (53.8%) of the *E. coli* isolates were possessing one of the SETC genes, *eaeA* but no other genes were detected (Table 1) [Dastmalchi and Ayremlou, 2012]. In their study, Mohammed *et al.,* (2019) also reported the presence of *eaeA* gene in few *E. coli* isolates and stx_1 gene in only one isolate detected from bovine faecal samples but no stx_2 and *ehxA* genes. Wani *et al.,* (2003) reported the presence of STEC (9.73%) possessing stx_1, stx_2 and *eaeA* from calf diarrhea cases from J&K, India which almost supports the findings of the current study. Shiga toxigenic *E. coli* possess the *eaeA* chromosomal gene which can produce intimin that causes the intimate attachment of the bacteria to the host cell aggravating their pathogenic property resulting into diarrhoea in young animals and also in human beings (Blanco *et al.,* 2004; Abbasi *et al.,* 2014).

All the ESBL producing *E.coli* isolates showed almost similar resistance/sensitivity pattern except in their antibiogram. All the isolates were resistant to cefepime, amoxyclav, ceftriaxone, ampicillin + cloxacillin, cefoperazone, cefotaxime, and ceftazidime. However, no resistances were observed against levofloxacin, amikacin, ampicillin + sulbactum, gentamicin, and doxycycline (Table 2). High levels of antibiotic resistance, as shown in this report, were also detected earlier by Banerjee and Acharyya (2020), Ibrahim *et al.,* (2016) and Hinthong *et al.,* (2017). Ali *et al.,* (2016) also found resistance against drugs like ampicillin (86.11%), amoxicillin-clavulanic acid (63.89%), cefotaxime (100%), ceftazidime (66.67%), tetracycline (72.22%) and gentamicin (61.11%) by ESBL *E. coli* pathogens in their study. Faruk *et al.,* (2016) reported that ampicillin, cefotaxime, ceftazidime, and cefuroxime (all 100%), tetracycline (93.54%) were highly resistant but imipenem (100%) to be highly sensitive to the ESBL *E. coli* strains isolated from cattle in their study which almost matches with the current findings. Resistance profile of the *E. coli* isolates in the present study reflected the usage pattern of antibiotics in the studied animals.

All the 13 ESBL producing strains were typed by RAPD-PCR using GTG5 gene to determine the genetic relationship among the strains. In RAPD-PCR, it was evident that the banding patterns of the isolates were the same with amplified fragment size ranging from 375bp to 1500bp was produced.

The phylogenetic analysis of ESBL producing strains after RAPD-PCR revealed that there are no differences among the isolates and was belonged to similar origin (data not shown). All isolates might come from the same type of infection and therefore possess similar genetic characters. These were also reported by Lim *et al.,* (2009)and Kar *et al.,* (2015) in their studies. The findings of Radu *et al.,* (2001) and Johnson *et al.,* (2003) are more or less similar to the current observations too.

The study, therefore, can be concluded as approx. 62% faecal samples from bovines diarrhoea cases screened in this study were positive for *E. coli*. All the isolates were positive to ESBL property with presence of bla_{CTX-M} (100%) and bla_{SHV} (84.6%) genes. Approx. 84.6% *E. coli* isolates were positive for ACBL property with presence of bla_{AmpC} gene. Pathogenic STEC gene (*eaeA*) was detected in approx. 54% *E. coli* strains found in this study which quite significantly is matching with the bovine diarrhoea outcomes.

The pathogenic *E. coli* isolates with ESBL property were quite resistant to many commonly used antimicrobials but few drugslike levofloxacin, amikacin, ampicillin + sulbactam, gentamicin, and doxycycline were effective against these pathogens *in-vitro*. These drug-resistant STECs may be of great clinical significance as these can easily create animal health hazards due to unsuccessful treatment with common antimicrobials and may be zoonotic too in few cases.

Conflict of Interest

The authors declare that there is no competing interest among the authors.

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