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Isolation and Molecular Characterization of Shigatoxigenic and Enteropathogenic *Escherichia coli* from diverse sources

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ABSTRACT

Total 45 *E. coli* isolates were recovered from faecal samples of 77 diarrhoeic and 85 healthy animals and birds, 51 milk samples and 48 diarrhoeic human stool samples. Multiplex PCR based molecular characterization targeting the virulence genes (*stx1*, *stx2, eae* and *bfpA*) could reveal presence of 24 Shiga toxin-producing *E. coli* (STEC) and 21 as enteropathogenic *E. coli* (EPEC). Among the STEC 19 isolates belonged to 13 different serogroups while four were untypable and one rough. Majority of STEC isolates carried *stx2* gene. Out of 21 EPEC isolates, 15 were serogrouped into 9 different serogroups and 6 were either untypable or rough. All the four EPEC isolates of milk origin belonged to serogroup O2. Only two isolates from dierrhoeic buffaloes were found to be typical.

Keywords: Animal, diarrhoea, EPEC, human, milk, STEC

Escherichia coli is primarily an enteric pathogen causing diarrhoea and associated diseases in both man and animals. Shiga toxin-producing *E. coli* (STEC) and enteropathogenic *E. coli* (EPEC) represent two of the six different categories of diarrhoeagenic *E. coli* recognized till date (Kaper *et al.* 2004). The morbidity and mortality associated with several outbreaks of STEC indicate the threat posed by these organisms to public health (Paton and Paton, 1998). STEC possesses two main virulence factors, Shiga toxin 1 and 2 encoded by stx_1 and stx_2 genes, respectively. The other virulence associated factor, intimin, encoded by *eae*, is responsible for intimate attachment of STEC to the enterocytes causing attaching and effacing (A/E) lesions in the intestinal mucosa. EPEC strains are defined as intimin-containing diarroeagenic *E. coli* that possess the ability to form A/E lesions on intestinal cells but do not possess *stx* genes (Knutton *et al.* 1989). EPEC strains are further classified as typical and atypical depending upon the presence or absence of bundle forming pili encoded by *bfpA* gene. *bfpA* positive strains are involved in bacteria–bacteria interaction and micro-colony formation but their role in cell adhesion remains unclear (Cleary *et. al.* 2004). Isolation of STEC and EPEC from different animals, birds and human have been reported from various parts of India (Wani *et al.* 2004; Dutta *et al.* 2011). However, little information is available on STEC and EPEC from the Noth Eastern region, especially Assam. Therefore, the present study was undertaken to isolate and characterize STEC and EPEC from animals, birds and human.

MATERIALS AND METHODS

Isolation

In the present investigation, a total of 318 samples consisting of faecal samples from 77 diarrhoeic animals (cattle-8, buffalo-11, sheep-8, goat-10, pig-18, dog-17 and elephant calf-5), 85 apparently healthy animals (cattle-12, buffalo-21. Sheep-16, goat-12, pig-7 dog-9 and elephant calf-8), cloacal swabs from 57 layer birds (diarrhoeic-32

and healthy-25), 51 milk samples from apparently healthy cows and 48 stool samples from human with diarrhoea were examined. The age of cattle, buffalo, sheep and goats were 0-6 months and the age of pigs and dogs were 1-6 weeks and 1-2 years, respectively. Cloacal swabs were taken from the birds. The samples were collected in sterile test tubes/vials and immediately brought to the laboratory under chilled conditions for processing.

The samples were immediately plated onto MacConkey's agar and incubated overnight at 37°C. From each sample, four to five suspected *E. coli* colonies based on colony morphology and staining reaction, were chosen and confirmed by standard morphological and biochemical tests as per the method described by Edwards and Ewing (1986). The *E. coli* isolates were serogrouped based on their somatic (O) antigen at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, India.

Multiplex Polymerase Chain Reaction

Bacterial DNA was extracted as per the procedure described earlier (Wani *et al.* 2009). A multiplex PCR (m-PCR) was carried out using 3 sets of oligonucleotide primers for *stx1*, *stx2*, and *eae* genes (Table 1). The PCR assay was carried out in 25.0 µl reaction volume containing 12.5 µl mastermix (Dream taq green master-mix, Fermentas, USA), 0.5 µl of each primer (0.5 µM), 1 µl of template DNA and 10.5 µl of nuclease free water. The samples were subjected to following PCR cyclying conditions: an initial denaturation at 96°C for 4 min, followed by 35 cycles of denaturation at 95°C for 20 sec, primer annealing at 57°C for 20 sec and extension at 72°C for 1 min and final extension at 72°C for 7 min. To ascertain the typical or atypical isolates, all EPEC isolates were examined for *bfpA* gene (Videl *et al.* 2004). The PCR reaction mixture and cycling conditions were same as mentioned above. Primer sequences are depicted in Table 1. A known STEC strain positive for *stx1*, *stx2* and *eae* (Fig:1) maintained in Department of Microbiology, College of Veterinary Science, AAU, Khanapara and EPEC strain positive for *bfpA* gene by Dr. T. Ramamurthy, NICED, Kolkata were used as positive controls, while MTCC 738 *E. coli* strain obtained from Institute of Microbial Technology, Chandigarh was used as negative control in PCR assays. Amplified PCR products were analysed by gel electrophoresis in 1.8% agarose gel containing ethidium bromide (0.5µg/ml) and were visualized by a gel documentation system (Kodak, Biostep, Germany).

RESULTS AND DISCUSSION

In the present study, 24 (7.55%) STEC isolates belonging to 13 different serogrups (O2, O4, O9, O22, O25, O43, O60, O69, O75, O91, O95, O130 and O162) could be recovered from various samples screened (Table 2). Among the STEC, 12 (15.58%) were isolated from cattle, buffalo, sheep, goat, pig and poultry with diarrhoea, while 9 (10.59%) from healthy cattle, buffaloes, sheep and dog. In a similar study, Baruah *et al.* (2011) could record STEC in 11.6% cattle, buffalo, goat, pig and dog with diarrhoea in Assam. STEC is reported to be very rare in chicken. However, isolation of STEC from a broiler flock with

Table 1: Details of primers used for detection of virulence genes of STEC and EPEC

Primers	Sequence	Target	Primer conc. (µM)	Product size (bp)	Reference
stx1 F	CAG TTA ATG TGG TGG CGA AGG	*stx1*	0.5	348	Cebula *et al.* (1995)
stx1 R	CAC CAG ACA ATG TAA CCG CTG				
stx2 F	ATC CTA TTC CCG GGA GTT TAC G	*stx2*	0.5	584	
stx2 R	GCG TCA TCG TAT ACA CAG GAG C				
eae F	TCA ATG CAG TTC CGT TAT CAG TT	*eae*	0.5	482	Stacy-Phipps *et al.* (1995)
eae R	GTA AAG TCC GTT ACC CCA ACC TG				
bfp F	GGA AGT CAA ATT CAT GGG GGT AT	*bfpA*	0.5	300	Videl *et al.* (2004)
bfp R	GGA ATC AGA CGC AGA CTG GTA GT				

Table 2: Virulence gene profile of STEC isolates

Source	Status	Serogroup	No. of isolates	Virulence gene(s)
Cattle	Diarrhoeic	O43	1	*stx1* and *stx2*
		O95	3	stx2
		O130	1	*stx1* and *stx2*
	Healthy	O91	1	stx2
		UT	2	*stx2*
Buffalo	Diarrhoeic	O22	2	*stx1* and *stx2*
	Healthy	O69	1	*stx2* and *eae*
Sheep	Diarrhoeic	O2	1	stx1
		O75	1	*Stx2*
	Healthy	O25	2	stx2
		Rough	1	*stx2*
Goat	Diarrhoeic	O60	1	*stx2* and *eae*
Pig	Diarrhoeic	O162	1	*stx2*
Dog	Healthy	O91	1	stx2
		UT	1	*stx2*
Poultry	Diarrhoeic	UT	1	*stx1*
Milk	Normal	O4	1	stx1
		O153	2	*stx1*
Total isolates			**24**	

diarrhoea (Dutta *et al.* 2011) is in agreement with the present observation. Detection of three (5.88%) STEC in raw milk of apparently healthy cows during the study corroborates the findings of previous workers (Da Silva *et al.* 2001). They concluded that EPEC in raw milk might be due to faecal contamination of utensils used for milking or of milker's hand.

The STEC isolates recovered were belonged to 13 different serogroups and they were different from reported earlier in Assam (Baruah *et al.* 2011). However, Serogroups O2, O22, O25, O60 and O75 detected in this study were also detected in free ranging yak and sheep in Arunachal Pradesh (Bandyopadhyay *et al.* 2012). Serogroups O22 and O91 have been associated with HC and HUS in humans (Orden *et al.* 2002). Out of 24 STEC isolates recovered, majority of them (79.2%) possessed *stx2* either alone or in combination with *stx1* or *eae*. Only five isolates carried *stx1* alone while 4 isolates carried both *stx1* and *stx2*. Baruah *et al.* (2011) too detected *stx2* in majority of the isolates in Assam. In France also, the prominent toxin genotype of STEC strains of cattle and human origin was found to be *stx2* (Pradel *et al.* 2001). Contrary to these, majority of non- O157 STEC carrying *stx1* from cattle (71.4%) and buffalo (83.7%) was reported in Bangladesh (Islam *et al.* 2008).

Total 21 (6.6%) EPEC could be isolated in the present investigation, out of which 10 (12.98%) and three (3.5%) were from diarrhoeic and healthy animals (buffalo, sheep, goat, and dog), respectively (Table 3). Higher rate of isolation of EPEC from diarrhoeic animals is also reported in Kashmir, where Wani *et al.* (2004) could isolate only 2.74 per cent of EPEC from the faecal materials of diarrhoeic poultry in Kashmir but slightly high rate (9.25%) of isolation of EPEC from broiler chicken was reported Dutta *et al.* (2011) in Mizoram. This study revealed isolation of four (7.02%) EPEC isolates from milk samples. Detection of EPEC in raw milk of apparently healthy cows during the study corroborates the findings of earlier workers (Da Silva *et al.* 2001). The serotypes associated with EPEC isolated from different sources were found to be O2, O3, O14, O20, O25, O60, O90, O106 and O170. Association of serotype O26 and O45 with the EPEC isolated from

Table 3: Virulence gene profile of EPEC isolates

Source	Status	Serogroup	No. of isolates	Virulence gene(s)
Buffalo	Healthy	O3	2	*Eae*
	Diarrhoeic	O14	1	*eae* and *bfpA*
		O25	1	Eae
		O90	1	Eae
		Rough	1	*eae* and *bfpA*
		UT	1	*eae*
Sheep	Diarrhoeic	UT	1	Eae
Goat	Diarrhoeic	O20	1	Eae
		UT	2	*Eae*
Dog	Healthy	O170	1	*Eae*
	Diarrhoeic	O20	1	*eae*
	Diarrhoeic	O20	1	*eae*
Poultry	Healthy	O60	1	eae
		O160	1	eae
		UT	1	*eae*
Milk	Normal	O2	4	*eae*
	Total isolates		**21**	

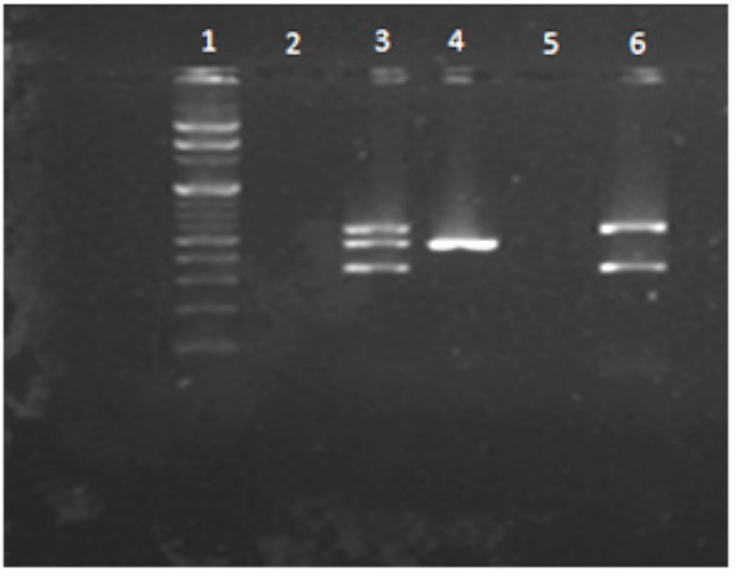

Fig. 1: Gel Picture of Multiplex PCR

Lane 1= 100 bp DNA ladder (Fermentus)
Lane 2= Negative control (NTC)
Lane 3= Positive control (548 bp, 482 bp, 348 bp)
Lane 4= Test isolate positive for *eae* (482 bp)
Lane 6= Test isolate positive for both *stx1* and *stx2* (348 bp, 584 bp)

diarrhoeic calves and lamb was also reported by Wani *et al.* (2009).

CONCLUSION

Non-O157 STEC and EPEC belonging to diverse serogroups are widely prevalent in different animal species. Majority of the STEC possessed *stx2* gene, which may pose a serious threat to human health being. Further, buffalo harbouring typical EPEC may act as a source of infection along with other animal species and human bearing typical and atypical EPEC..

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