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Molecular and phenotypic characterization of shigatoxigenic *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC) from piglets and infants associated with diarrhoea in Mizoram, India

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Limited information is available on shiga toxin producing *Escherichia coli* (STEC) in pigs and infants from Aizawl, Mizoram and North Eastern region of India. This cross sectional study was conducted on faecal samples from pigs and infants to detect and characterize of STEC and enteropathogenic *E. coli* (EPEC). Serogrouping, molecular and phenotypic characterizations were done by standard molecular and cytotoxic assays. Out of 48 *E. coli* strains isolated from 320 diarrhoeic fecal samples of piglets, 44 belonged to 18 different serogroups, 3 (6.25%) were untypeable (UT) and 1(2.08%) was recorded as rough strain (R). Similarly, out of 17 *E. coli* strains isolated from 264 diarrhoeic fecal samples of infants, 16 belonged to O60 (94.1%) and 1(5.88%) was untypeable. Virulence genes (*stx*1, *stx*2, *eaeA* and *hlyA*) were detected by multiplex PCR assay. A total of 1260 *E. coli* were isolated from piglets (720) and infants (540) from 584 faecal samples. All together, 5.16% (65) *E. coli* isolates were found to be positive for at least one virulence gene (6.66% piglets and 3.15% infants). Out of the virulent gene positive *E. coli* 3.17% (32 from piglets and eight from infants) and 1.98% (16 from piglets and nine from infants) were recorded as STEC and EPEC, respectively. On the other hand, from the total 2.14% stx_2 positive isolates, 16 and 11 were positive for stx_{2e} and stx_{2c} subtypes, respectively. Similarly, from the 4.04% eaeA positive isolates, 1.19% (15) were positive for *bfpA* gene, of which 1.67% (12) were piglets and 0.60% (3) were infants. All the isolates were exhibited varying degree of CPE on vero cell lines. In conclusion, STEC and EPEC seem to be associated with diarrhoea in piglets and infants in Mizoram. In piglets STEC strains represent as a major cause of diarrhoea while EPEC strains represent as major cause of diarrhoea in infants in North Eastern region of the India.

Key words: Enteropathogenic *E. coli* (EPEC), infants; piglets, shigatoxigenic *E. coli* (STEC), vero cell cytotoxicity.

INTRODUCTION

Pathogenic *Escherichia coli* are one of the most important groups of bacteria causing diarrhoea and extraintestinal infections in human and animals (Levine, 1987). Major diseases caused by *E. coli* are piglet diarrhoea (Fair brother et al., 2000), oedema disease (Chen et al., 2004) and postweaning diarrhoea in pigs

(Choi et al., 2001). In human, it causes infant diarrhoea, thrombotic thrombocytopenia purpurea (Paton and Paton, 2005), haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (Nataro and Kaper, 1998; Leelaporn et al., 2003; Paton and Paton, 2005; Zweifel et al., 2006). Shigatoxin producing *E. coli* (STEC), also known as verotoxin producing *E. coli* (VTEC), comprises a serologically diverse group of pathogens that cause disease in humans and animals (Barman et al., 2008). STEC constitute one of the most important causes of foodborne diseases worldwide (Wani et al., 2004). The common features of STEC are the production of shigatoxins (Stx) that are considered to be the major virulence factors. Two major groups of Stxs are named as stx_1 and stx_2; where the former one is nearly identical to the toxin of *Shigella dysenteriae* type 1. stx_2 shares less than 60% amino acid sequence with stx_1 (Barman et al., 2008).

EPEC isolates are defined as an intimin containing diarrhoeagenic *E. coli* that possess the ability to form attaching and effecting lesions on intestinal cells and that do not possess genes coding for Shiga toxins (Kaper, 1996; Bhat et al., 2008). EPEC isolates are leading cause of diarrhoea, especially among infants in the developing world (Bhat et al., 2008).

Diarrhoeagenic *Escherichia coli* (STEC and EPEC) are responsible for an estimated 300,000 to 500,000 death annually in children under the age of five years (Fleckenstein et al., 2010). STEC and EPEC are commonly recovered from the faeces of food producing animals and pose threats to health of humans and livestock. The majority of the populations in north eastern region of India are traditionally dependent on pig rearing for their livelihood; and there are no taboos for consumption of meat. It is a common feature to observe that each and every rural family maintains a backyard piggery unit in this region. The density of pig population in North eastern region of India is highest in the country with Mizoram contributing 2.18×10^4 out of total 3.879×10^5 pig population in North eastern region of India (Statistics of animal population in NE India, 2003).

There is limited information available on incidence of STEC and EPEC in piglets and infants in India and particularly in North Eastern Region of India. The present study was undertaken with the objectives to study the level of STEC and EPEC in the piglets and infants in Mizorem state, to investigate the association of STEC and EPEC with diarrhoea of piglets and infants.

MATERIALS AND METHODS

Sampling and isolation of *E. coli* isolates

A total of 584 faecal samples were collected from 320 piglets (20 to 60 days old) and 264 infants (2 to 24 months old) with the history of diarrhoea from different districts of Mizoram during June, 2010 to April, 2011. Fecal samples were inoculated on Sorbitol MacConkey's agar (HiMedia, Mumbai, India) plates and incubated at 37°C for 24 h. Five randomly selected pink colored colonies were picked up from each plate and were again sub cultured on to Eosin Methylene Blue agar (HiMedia, Mumbai, India) plates to observe the characteristics metallic sheen of *E. coli*. The well separated single colonies were picked up on nutrient agar slants as pure culture and subjected to standard morphological and biochemical tests.

Serogrouping

All the *E. coli* isolates were serotyped on the basis of their "O" & "H" antigen. Somatic (O) typing was done at National *Salmonella* and *Escherichia* Center, Central Research Institute, Kasauli H.P. 173204 (India) while flagellar (H) typing was carried out by agglutination test using specific flagellar antisera (Denka Seiken, Japan) as per manufacturer's instruction with suitable modifications. Specific antisera were used for flagellar typing.

Preparation of bacterial DNA lysates

Each E. *coli* isolates were inoculated in 5 ml Luria Bertani (LB) (HiMedia, Mumbai, India) broth and incubated at 37°C overnight under constant shaking. After incubation, 1 ml of the bacterial broth culture was taken in a sterile microcentrifuge tube and pelleted at 8000 rpm for 10 min at 4°C. The bacterial pellet was washed thrice with sterile normal saline solution (NSS) (0.85% NaCl) and centrifuged at 8000 rpm for 10 min at 4°C and finally resuspended in 300 µl nuclease free sterile water (NFW). Bacterial suspension was boiled for 10 min in a boiling water bath followed by immediate chilling for 10 min. The bacterial lysate was centrifuged again at 5000 rpm for 5 min to sediment the cell debris and the supernatant was used as template DNA for PCR assay.

Detection of virulence genes for STEC and EPEC by multiplex PCR

A multiplex PCR was carried out using four sets of oligonucleotide primers for stx_1, stx_2, *eaeA* and *hlyA* genes as per the method described by Paton and Paton (1998) with suitable modifications. The detail of the primers used in this study is depicted in Table 1. The multiplex PCR mixture of 25 µl contained 2.5 µl of 10X PCR buffer with $MgCl_2$, 1 µl each primer within the four primer sets at a concentration of 250 nM, 2 µl of 10 mM each of dNTPs, 0.2 µl of 5.0 U of *Taq* DNA polymerase and 4.0 µl of template DNA and the rest is NFW (8.3 µl) to make up volume of 25 µl. The PCR reaction was performed in a thermal cycler (Thermo Electron, Germany) using following cycling conditions: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 s, primer annealing at 65°C for 45 s and extension at 72°C for 45 s, followed by a final extension at 72°C for 5 min. The amplified PCR products were analysed by agarose gel electrophoresis (1.5% agarose in 1X TAE) at 80 V/cm for 45 min and stained with ethidium bromide (0.5 µg/ml). The products were visualized under ultraviolet transilluminator and documented by a gel documentation system (AlphaImager). A known molecular weight marker (100 bp DNA ladder) was used for each run to compare the amplicon size. The PCR reaction was performed three times to ensure the repeatability of the techniques and to make sure that strains were correctly assigned to respective patterns.

Multiplex PCR for subtyping of Stx_2 encoding genes using specific primers

All the stx_2 positive STEC isolates were further tested for their

Table 1. Details of the oligonucleotide primers used in the present study.

Primer name	Sequences (5`- 3`)	Expected amplicon size (bp)	References
stx_1 F	ATAAATCGCCATTCGTTGACTAC	180	Paton and Paton (1998)
stx_1 R	AGAACGCCCACTGAGATCATC		
stx_2 F	GGCACTGTCTGAAACTGCTCC	255	Paton and Paton (1998)
stx_2 R	TCGCCAGTTAATCTGACATTCTG		
stx_2c F	GCGGTTTTATTTGCATTAGT	124	Paton and Paton (2005)
stx_2cR	AGTACTCTTTTCCGGCCACT		
stx_2d F	GGTAAAATTGAGTTCTCTAAGTAT	175	Paton and Paton (2005)
stx_2d R	CAGCAAATCCTGAACCTGACG		
stx_2e F	ATGAAGTGTATATTGTTAAAGTGGA	303	Paton and Paton (2005)
stx_2e R	AGCCACATATAAATTATTTCGT		
stx_2f F	TGTCTTCAGCATCTTATGCAG	150	Paton and Paton (2005)
stx_2f R	CATGATTAATTACTGAAACAGAAAC		
eaeA F	GACCCGGCACAAGCATAAGC	384	Paton and Paton (1998)
eaeA R	CCACCTGCAGCAACAAGAGG		
hlyA F	GCATCATCAAGCGTACGTTCC	534	Paton and Paton (1998)
hlyA R	AATGAGCCAAGCTGGTTAGCT		
bfpA F	GATTGAATCTGCTCTGGATTGA	426	Wieler et al. (1996)
bfpA R	GGATTACTGTCCTCACATAT		

F = Forward primer; R= reverse primer.

subtypes (*stx_2c, stx_2d, stx_2e and stx_2f*) encoding genes using specific primer sets as described by Wang et al. (2002) with suitable modifications. The PCR reaction was carried out in a 0.2 ml thin wall PCR tubes using the reaction mixture as described above for each isolates with a final volume of 25 µl except the concentration of each primer was 500 nM. PCR was carried out in a thermal cycler and the cycling condition consisted of initial denaturation at 95°C for 8 min followed by 30 cycles of amplification with denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, ending with a final extension at 72°C for 7 min. The amplified PCR products were analyzed by agarose gel electrophoresis (1.5% agarose in 1X TAE) at 80 V/cm for 45 min and the gels were then stained with ethidium (0.5 µg/ml) and data was documented with gel documentation system (AlphaImager). A 100 bp DNA ladder was used as marker for band interpretation (Jomezadeh et al., 2009).

PCR for detection of bfpA gene

All the *eaeA* positive isolates were further subjected to PCR for the detection of *bfpA* gene (426 bp) using *bfpA* specific primer as described by Wieler et al. (1996) with suitable modifications. PCR reaction was carried out in a 0.2 ml thin wall PCR tubes with the final volume of 25 µl reaction mixture with the 2.5 µl of 10X PCR buffer (with $MgCl_2$), 2.0 µl dNTP's (10 mM), 1 µl of bfpA forward and reverse primer (500 nM), 0.2 µl of Taq polymerase (5 U/µl), 4 µl of DNA lysate and the rest is NFW (14.3 µl) to make up volume of 25 µl. PCR assay was carried out in a thermal cycler with the cycling condition consisting of initial denaturation step (94°C for 5 min) and followed by 30 cycles of amplification with denaturation at 94°C for 1 min, primer annealing at 57°C for 45 s, extension at 72°C for 1.2 min and a final extension at 72°C for 5 min. The amplified PCR products were analyzed by agarose gel electrophoresis (1.5% agarose in 1X TAE) at 80 V/cm for 45 min and stained with ethidium bromide (0.5 µg/ml). The products were visualized with UV trans-illuminator and imaged with gel documentation system (AlphaImager). A known molecular weight marker (100 bp DNA ladder) was also used for each run to compare the amplicon size (Rugeles et al., 2010). All the PCR were performed three times to record the repeatability of the result. Samples with repeated result in all the three occasions were recorded as either positive or negative.

Phenotypic characterization of STEC

All the STEC isolates were further characterized phenotypically by observing the cytotoxic effect on vero cell line as per the method described by OIE Terrestrial Manual (2008) as well as El Sayed Zaki and El-Adrosy (2007) with suitable modifications. Vero cell line was procured from NCCS, Pune and maintained by periodic sub culturing in laboratory.

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Abbreviations: HC, Haemorrhagic colitis; **HUS,** haemolytic uraemic syndrome, **STEC,** shigatoxigenic *E. coli*; **EPEC,** enteropathogenic *E. coli*.

Table 2. Virulence genes profile of *E.coli* isolated from diarrhoeic piglets.

Serogroup	Number of isolates	*stx*$_1$	*stx*$_2$	*eaeA*	*hlyA*
O9	2	-	+	-	-
O20	2	-	-	+	-
O24	2	+	-	-	-
O59	1	-	+	+	+
O60	1	-	+	+	-
O85	8	-	-	+	+
O100	3	-	+	-	+
O103	3	+	+	+	+
O112	6	-	-	+	+
O113	3	+	+	-	+
O116	2	+	-	-	+
O118	2	+	-	-	-
O119	2	-	+	+	+
O123	3	-	-	+	-
O137	2	-	+	+	-
O152	2	-	+	+	+
UT	3	-	-	+	+
Rough	1	+	-	-	+
Total	48	13	19	34	35

+ = Positive; - = Negative; UT = untypable.

Procedure for vero cell cytotoxicity assay

Colony sweeps of the STEC isolates were inoculated in Penassay broth (antibiotic medium 3 supplemented with bacitracin) (HiMedia, Mumbai, India). After inoculation, the isolates were allowed to grow by overnight incubation at 37°C and centrifuged the fluid cultures at 10,000 x *g* for 10 min. Supernatants obtained after centrifugation were filtered through 0.2 µm pore size membrane filters. The cell monolayer was washed once with the maintenance medium (DMEM, Hyclone) and volumes (50 µl) of the filtrates were applied to confluent vero cell monolayers (in 12 well plate). Cytopathic effects were evaluated after 72 h of incubation at 37°C maintained with a constant supply of 5% CO_2. Rounding of 50% or more of the cells as well as the cell sheets becoming disintegrated with blackening were considered as positive.

Data management and analysis

Data collected during sampling and laboratory analysis were entered and stored in MS Excel spreadsheet. Data were thoroughly scrutinized for errors and proper coding before subjected to statistical analysis and analyzed using SPSS soft ware version 17.0.

RESULTS

Out of 48 *E. coli* strains isolated from 320 diarrhoeic fecal samples of piglets, 44 belonged to 18 different sero-groups, 3 (6.25%) were untypeable (UT) and 1(2.08%) was recorded as rough strain (R) (Table 2). Out of total 18 somatic groups, 0.85 was found in highest number (8; 16.6%) followed by O112(6; 12.5%), O103(3; 6.25%), O123(3; 6.25%), O100(3; 6.25%), O113(3; 6.25%), O9(2; 4.16%), O24(2; 4.16%), O20(2; 4.16%), O119(2; 4.16%), O137(2; 4.16%), O116(2; 4.16%), O152(2; 4.16%), O118(2; 4.16%), O60(1; 2.08%) and O59(1; 2.08%). Similarly, out of 17 *E. coli* strains isolated from 264 diarrhoeic fecal samples of infants, 16 belonged to O60 (94.1%) and 1(5.88%) was untypeable (Table 3). Out of total 65 *E. coli* strains isolated from both piglets and infants, 50 were subjected to flagellar typing (40 from piglets and 10 from infants) after checking their motility test by hanging drop method and Craiege's tube experiment. 15 E. *coli* isolates (8 from piglets and 7 from infants) were found to be non- motile and were recorded as H^- serotype. Four flagellar types were recorded in infants: H2 (3, 17.6%; typical EPEC), H27 (5, 29.41%; atypical EPEC), H21 (2; 11.76%) and H^- (41.2%). Similarly, 6 flagellar types were recorded in piglets: H4 (10, 25%), H6 (8, 20%), H10 (6, 15%), H19 (9, 22.5%), H40 (7, 17.5%) and H- (16.7%).

A total of 1260 *E. coli* colonies were isolated from the 584 faecal samples. Of 1260 *E. coli* isolates (720 from piglets and 540 from infants) tested for four virulence genes (*stx*$_1$, *stx*$_2$, *eae*A and *hly*A), 5.16% (65) carried at least one virulence gene. 6.67% (48) from piglets were classified under 11 genotypic profiles (Table 2, Figure 1). It was also found that 0.55% (4), 0.27% (2), 0.83% (6), 0.41% (3), 0.41% (3), 0.69% (5), 0.69% (5), 1.52% (11), 0.41% (3), 0.41% (3) and 0.41% (3) were carried in the pattern of *stx*$_1$ only, *stx*$_2$ only, *stx*$_1$ + *stx*$_2$, *stx*$_2$ + *eaeA* , *stx*$_2$ + *hlyA, stx*$_2$ +*eaeA* + *hlyA, eaeA* only, *eaeA* + *hlyA*,

Table 3. Virulence genes profile of *E. coli* isolated from diarrhoeic infants.

Serogroup	Number of isolates	*stx*$_1$	*stx*$_2$	*eaeA*	*hlyA*
O60	1	-	+	+	-
O60	1	-	-	+	-
O60	1	-	+	+	-
O60	1	-	-	+	-
O60	1	-	-	+	-
O60	1	-	+	+	-
O60	1	-	-	+	-
O60	1	-	+	+	-
O60	1	-	-	+	-
O60	1	-	+	+	-
O60	1	-	+	+	-
O60	1	-	-	+	-
O60	1	-	+	+	-
O60	1	-	-	+	-
O60	1	-	+	+	-
O60	1	-	-	+	-
UT	1	-	-	+	-
Total	17	0	8	17	0

+ = Positive; - = Negative; UT = untypable.

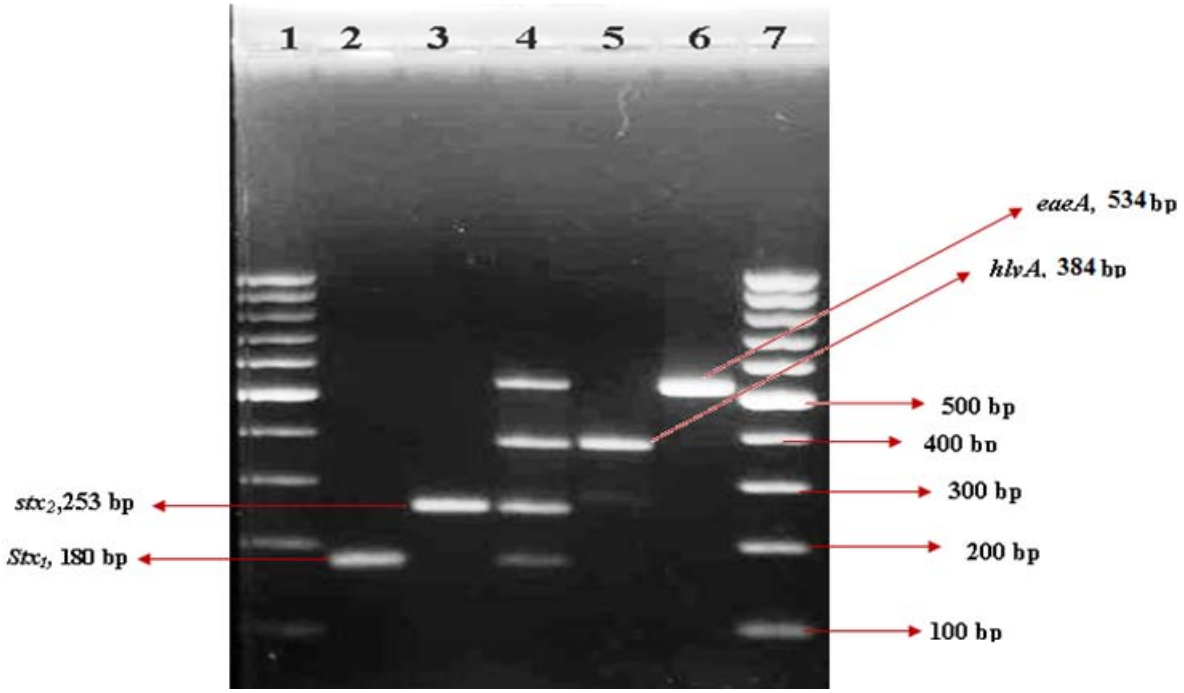

Figure 1. Multiplex PCR analysis of *E. coli* isolates for four virulence genes (*stx*$_1$, *stx*$_2$, *eaeA* and *hlyA*). Lane 1 and 7: 100 bp DNA ladder, Lane 2: *E. coli* isolate positive for *stx1* (180 bp), Lane 3: *E. coli* isolate positive for *stx2* (255 bp), Lane 4: positive JK1 control, Lane 5: *E. coli* isolate positive for *eaeA* (384 bp), Lane 6: *E. coli* isolate positive for *hlyA* (534).

stx$_1$ + *stx*$_2$ + *eaeA* + *hlyA*, *stx*$_1$ + *hlyA* and *stx*$_1$ + *stx*$_2$ + *hlyA*, respectively (Table 4). Of the total 2.14% (27) *stx*$_2$ positive *E. coli* isolates (19 from piglets and 8 from infants) subjected to subtyping by using specific primers (*stx*$_2$*c*, *stx*$_2$*d*, *stx*$_2$*e* and *stx*$_2$*f*), 1.80% (13) and 0.83% (6) from a total of 2.64% (19) were found to be positive for

Table 4. Distribution of virulence genes in *E. coli* isolated from piglets from different districts of Mizoram.

Virulent gene	No. of *E. coli* isolates and their percentage
stx$_1$ only	4 (0.55%)
stx$_2$ only	2 (0.27%)
stx$_1$ and *stx*$_2$	6 (0.83%)
stx$_2$ and *eaeA*	3 (0.41%)
stx$_2$ and *hlyA*	3 (0.41%)
stx$_2$, *eaeA* and *hlyA*	5 (0.69%)
eaeA only	5 (0.69%)
eaeA and hlyA	11(1.52%)
stx$_1$, *stx*$_2$, *eaeA*, and *hlyA*	3 (0.41%)
stx$_1$ and *hlyA*	3 (0.41%)
stx$_1$, *stx*$_2$, and *hlyA*	3 (0.41%)
Total	48 (6.67%)
STEC	32 (4.44%)
EPEC	16 (2.22%)

Similarly, out of the 3.14% (17) *E. coli* isolated from infants, 1.48% (8) were STEC and 1.66% (9) were EPEC. All the isolates were categorized into 2 genotypic profiles (Tables 3 and 5), where 1.48% (8) and 1.66% (9) were carried in *stx*$_2$ + *eaeA* and *eaeA* only, respectively.

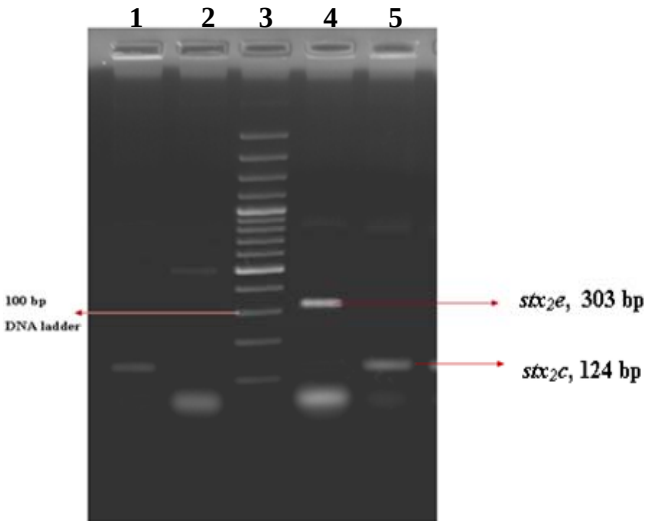

Figure 2. Subtyping of *stx*$_2$ positive *E. coli* isolates by PCR using different subtypes (stx$_2$c, stx$_2$d, stx$_2$e and stx$_2$f) specific primers. Lane 1: *E. coli* isolate positive for stx$_2$c (124 bp), Lane 2: *E. coli* isolate negative for stx$_2$ subtypes, Lane 3: 100 bp marker of DNA ladder, Lane 4 : *E. coli* isolate positive for stx$_2$e (303 bp), Lane 5: *E. coli* isolate positive for stx$_2$c (124 bp).

stx$_2$*e* and *stx*$_2$*c* subtype, respectively in piglets (Figure 2 and Table 5). Similarly, out of the total 1.48% (8) *stx*$_2$ positive *E. coli* isolates from infants, 0.93% (5) and 0.56% (3) were positive for *stx*$_2$*c* and *stx*$_2$*e* subtypes, respectively. Of the total 4.04% (51) *eaeA* positive E. *coli* isolated (34 from piglets and 17 from infants), 29.41% (15)

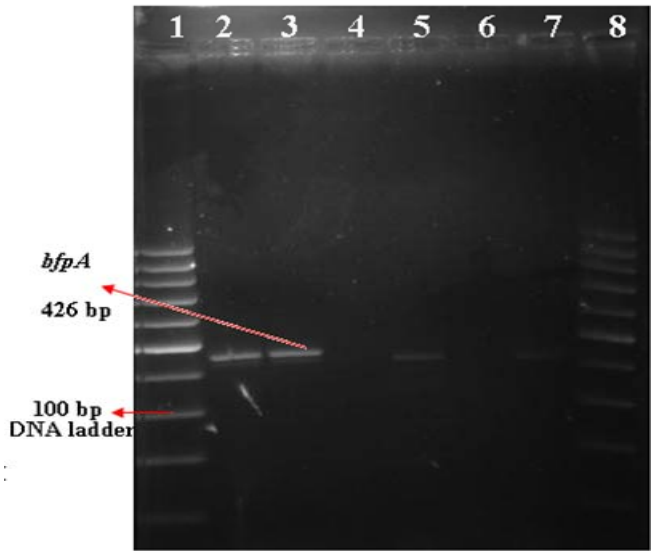

Figure 3. bfpA specific PCR for detection of *bfpA* gene in *eaeA* positive *E. coli* isolated from piglets and infants. Lane 1: 100 bp DNA ladder; Lane 2: *E. coli* isolate positive for *bfpA* (426 bp); Lane 3: *E. coli* isolate positive for *bfpA* (426 bp); Lane 4: *E. coli* isolate negative for *bfpA*; Lane 5: *E. coli* isolate positive for *bfpA* (426 bp); Lane 6: *E. coli* isolate negative for *bfpA;* Lane 7: *E. coli* isolate positive for *bfpA* (426 bp); Lane 8: 100 bp DNA ladder.

Table 5. Distribution of virulence genes in *E. coli* isolated from infants in different hospitals of Aizawl, Mizoram.

Virulent gene	No. of *E. coli* isolated
stx$_1$ only	-
stx$_2$ only	-
stx$_1$ and *stx*$_2$	-
stx$_2$ and *eaeA*	8 (1.48%)
eaeA only	9 (1.66%)
Total	17 (3.14%)
STEC	8 (1.48%)
EPEC	9 (1.66%)

(15) *E. coli* isolates were found to be positive for *bfpA* gene with an amplicon size of 426 bp, of which, 23.53% (12) were from piglets and 5.88% (3) were from infants (Figure 3).

A total of 40 STEC isolates (8 from infants and 32 from piglets) were subjected to vero cell cytotoxicity assay. All the isolates exhibited characteristic cytotoxic effect (Figure 4a to d).

DISCUSSION

Pathogenic *E. coli* are one of the most important groups of bacteria causing diarrhoea and extraintestinal infections in human and animals (Levine, 1987). The

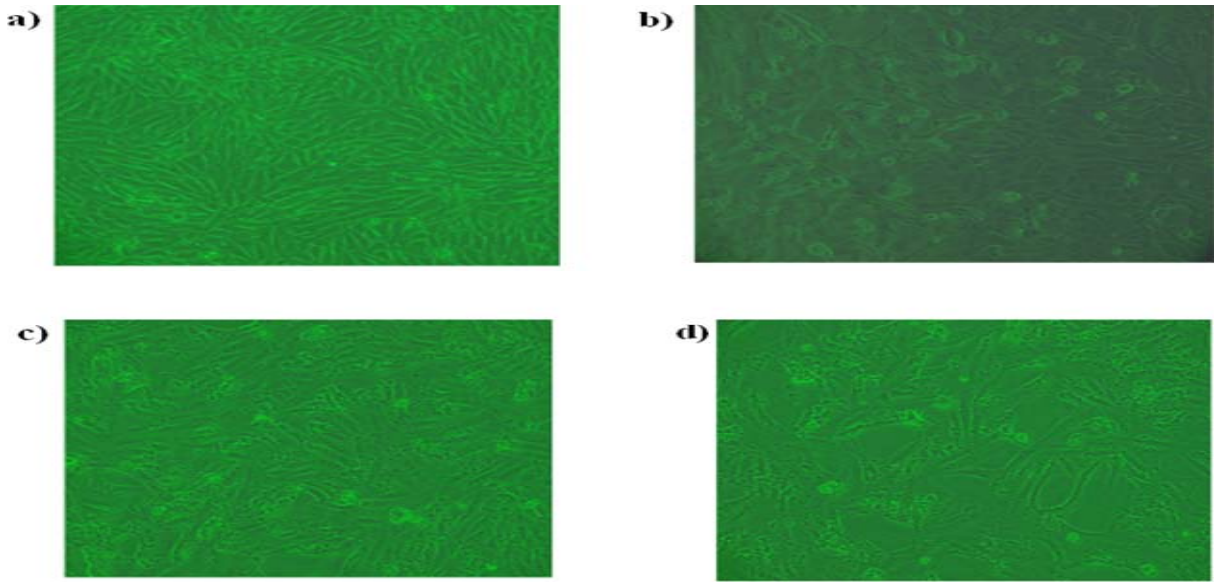

Figure 4. a) Confluent monolayer of healthy vero cells; **b)** CPE in verocells after 12 h of incubation with filtrate extracts of STEC isolated from piglets and infants (200X); **c)** CPE in vero cells after 24 h of incubation (200X); **d)** CPE in vero cells after 48 h of incubation (200X).

present study identifies different types of serotypes of *E. coli*. Remarkably large number of O85, 18.2% (8) serogroup recorded from piglets in the present study may be an indication of potential newly emerging pathogenic serogroup associated with diarrhoea in piglets in this region of India. It may pose threat to human infections in future as pigs are one of the major reservoirs of STEC. Most of the oedema disease causing STEC in pigs belongs to serogroup O8, O138, O139, O141, O147 and UT (Parma et al., 2000; Helgerson et al., 2006; Barman et al., 2008). Most of the diarrhoea causing STEC and EPEC in piglets belong to the serogroups O8, O9, O64, O101, O149, O162 (Parma et al., 2000), while most of the post weaning diarrhoea (PWD) causing STEC and EPEC in pigs belong to the serogroups O8, O138, O141, O147, O149 and O157 (Frydendahl, 2002), but Chen et al. (2004) reported that O9, O11, O20, O32, O91, O101, O107, O115, O116 and O131 as some of the novel serogroups emerged as PWD causing pathogens on pigs in some regions of China.

In case of infants, the predominant serotype recorded in this study was 060 and 94.11% (16) followed by UT, 5.88% (1). In case of infants, Pandey et al. (2003) reported O86 and O138 serogroups as pathogenic EPEC detected from patients with acute diarrhea at Kolkata, India. Rivero et al. (2010) reported O157, O145, O126, O121, O111, O118 serogroups of STEC and EPEC isolated from infants suffering from diarrhea in Argentina. In the present study O60 (n =16) was recorded as only valid serogroup. Till date, no valid data is available related with infant diarrhoea in this region of India. Although, the sample size is too low, but with the available data from the present study, O60 may be considered as the newly emerging pathogenic *E. coli* responsible for diarrhoea in infants in Mizoram. O60 serogroup was detected from both piglets 2.08% (1) and infants 94.11% (16), which suggests that there might be possibility of transmission of infection between piglets and infants. But it will be too early to mention that, common pathogenic *E. coli* are interchanging their host in this region of India. A further large scale study is required to put any valid conclusion in this aspect.

The serogrouping based on H antigen is in line with different authors throughout the world. Rivero et al. (2010) reported flagellar types H7, H^-, H11, H19 and H2 in diarrhoeic infants, whereas Ge et al. (2002) reported flgellar types H11, H7 and NM in diarrhoeic infants. A wide range of of flagellar types in diarrhoeic and non diarrhoeic infants were also reported by various others workers (Rehua-Mangia et al., 2004; Bettelheim et al., 2003; Jenkins et al., 2003). Schierack et al. (2006) recorded the prevalence of flagellar types H6, H^-, H10 and H4 in pigs.

The present study clearly indicates that the prevalence of stx_2 gene in STEC isolates were higher than stx_1 gene. This finding is in line with the works of different authors in India and international who have got higher prevalence of stx_2 gene in piglets with and without diarrhoea (Kataria, 2009; Parma et al., 2000; Helgerson et al., 2006). Therefore, presence of higher number of stx_2 (22/48) than stx_1 (16/48) indicate the magnitude of virulence in both pig and human and also indicating the possibility of transmission of such organisms between pig and human beings due to close association. In case of piglets, the

present study found that 18.75% (9) of STEC isolates harbored both *stx*$_1$ and *stx*$_2$ genes, which is higher than the result obtained by Kataria (2009) in piglets in the same region. Bhat et al. (2008) recorded 41.71% STEC harboring both *stx*$_1$ and *stx*$_2$ genes in lambs but not in piglets. These variations might be because of the species differences as well as health condition of the animals, from where samples were being collected. In the present study, out of 16 EPEC isolates, 68.75% (11) carried both *eaeA* and *hlyA* genes, while 31.25% (5) carried only *eaeA* gene. Kataria (2009) reported 80.95% (17) EPEC isolates carried *eaeA* and *hlyA* and 19.08% (4) EPEC isolates carried only *eaeA* gene.

Ghosh and Ali (2010) reported a total of 17 typical and atypical EPEC from 396 children with and without diarrhoea. The present study also supports the opinion that Shiga toxin-producing *E. coli* does not pose a major threat to human health in India. Prevalence of *eaeA* gene was higher than *stx*$_2$ gene in infant isolates in the present study. Enteropathogenic *E. coli* (EPEC) was first recognized as a cause of infantile diarrhoea in the 1940s, and was associated with outbreaks in hospitals and nurseries in most of the developing countries (Trabulsi et al., 2002; Blanco et al., 2006b; Aslani and Alikhani, 2009).

STEC and EPEC are commonly recovered from the faeces of food producing animals and pose threats to health of humans and livestock (Bhat et al., 2008; Nataro and Kaper, 1998). STEC is an emerging pathogen, which is recognized as an important cause of human sporadic and epidemic diarrhoea, hemorrhagic colitis and HUS (Gavin and Thomson, 2004). EPEC isolates are leading cause of diarrhoea, especially among infants in the developing world (Bhat et al., 2008). In India, there is a paucity of information on STEC and it has not been identified as a significant etiological agent of diarrhoea for humans (Wani et al., 2004). Till date, sporadic reports are available on isolation, identification and characterization of STEC in human and animals (Wani et al., 2004; Barman et al., 2008). Information on prevalence of STEC and EPEC in pigs is rare in India. Only few reports are available on association of STEC and EPEC in pigs and till date no reports are available for infants in this region (Mizoram) of the country. In Mizoram, pork is the major source of animal protein and people of this hilly state rear about 5 to 10 pigs near their residence and share the common source of water. Therefore, chances of getting infection of STEC and EPEC through contaminated water are very high.

Till date only a few variants of stx$_1$ and more than 20 variants of stx$_2$ have been reported (Gourmelon et al., 2006; Hussain et al., 2007). Bastian et al. (1998) reported that stx$_2$e production is specific to strains pathogenic for pigs. Many studies showed that *stx*$_2$ and *eaeA* of *E. coli* were associated with oedema disease in pigs (Wani et al., 2006). Barman et al. (2008) revealed that *stx*$_2$*e* bearing haemolytic *E. coli* was the prime cause of oedema disease in pigs in Assam state of India.

Schierack et al. (2006) reported higher prevalence of *stx*$_2$*e* positive *E. coli* isolates obtained from diarrhoeic piglet than from clinically healthy piglets. Wang et al. (2002) reported various subtypes of stx$_2$ with stx$_2$c being the highest prevalent subtype in human. Some stx variants such as stx$_2$c and stx$_2$d act are associated with an increased risk of developing HC and/or HUS (Paton et al., 1998; Friedrich et al., 2002). Wani et al. (2006) reported stx$_1$c as major prevalent subtype in calves and lambs. Zweifel et al. (2006) reported that stx$_2$e producing *E. coli* isolates from human and pigs differ in their virulence profiles and interactions with intestinal epithelial cells.

Blanco et al. (2006a) detected 5.2% (105) atypical EPEC strains (*eae*+ and *bfp*-) and 0.2% (5) typical EPEC (*eae*+ and *bfp*+) in 2015 patients investigated in Spain. Wani et al. (2006) recorded 23 *E. coli* isolates as EPEC carrying *eaeA* gene out of 326 *E. coli* isolates. Of the 23 EPEC isolates, majorities (78.26%) were recorded as atypical and 21.73% (5) were recorded as typical EPEC. Dhanashree and Mallya (2008) in Manglore, India reported that out of 110 *eaeA* positive *E. coli* isolates only 1 which was found to be positive for *bfpA*. Ghosh and Ali (2010) in Delhi, India recorded a total of 17 typical and atypical EPEC from 396 children with and without diarrhoea. Typical EPEC strains possess the bundle-forming pilus (*bfpA*) gene and have been almost exclusively isolated from humans and are well recognized as a cause of gastroenteritis in infants (Nataro and Kaper, 1998). Meanwhile, atypical EPEC do not harbor the *bfpA* gene and have been isolated from different animal species (Nataro and Kaper, 1998; Trabulsi et al., 2002; Corte´s et al., 2005).

Rivero et al. (2010) reported the confirmation of VT production in children associated with acute diarrhoea in Argentina by cytotoxicity assay on vero cell monolayers. Similar kind of observation was also reported by other worker (Leelaporn et al., 2003). The profound cytotoxicity of Stx to vero cells remains the 'gold standard' for confirmation of putative Stx-producing isolates (Paton and Paton, 1998; 2005; Cermelli et al., 2002). While the test is sensitive, it is not available in most routine diagnostic laboratories because it is labour intensive and results can take three to four days after the cell culture is inoculated. Therefore, molecular techniques such as PCR should be preferred over cytotoxicity assay for detection of STEC in future studies, as PCR is sensitive, specific and can provide effective results within few hours, but for effective confirmation of STEC, both PCR and cytotoxicity assay should be preferred.

Conclusion

In conclusion, the present study found out that STEC strains were found to be a major cause of diarrhoea in piglets while EPEC strains in infants in Mizoram state of India. The presence of higher number of *stx*$_2$ (22/48) than

stx$_1$ (16/48) indicate the highest magnitude of virulence in both pig and human and also indicating the possibility of transmission of such organisms between pig and human beings due to close association. Both PCR and Cyto-toxicity assays are required for confirmation of STEC diagnosis.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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