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Detection and Molecular Characterization of Shiga Toxin Producing *Escherichia coli* and Enteropathogenic *Escherichia coli* from Piglets in Mizoram, India

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A total of 100 fecal samples from less than 3 month old piglets with (n=60) or without (n=40) diarrhoea were collected and processed for isolation of *Escherichia coli* and screened for the presence of *E. coli*. A total of 254 *E. coli* isolates obtained were subjected to multiplex polymerase chain reaction (m-PCR) based screening of stx_1, stx_2, *eae*A and *hly*A genes. Out of 254 isolates, presence of at least one virulence gene were detected in 51 (20.08%) isolates of which 30 (11.81%) and 21 (8.26%) isolates were identified as shiga toxin producing (STEC) and enteropathogenic *E. coli* (EPEC), respectively. The antimicrobial sensitivity pattern of the STEC and EPEC isolates obtained in the present study revealed prevalence of high level of resistance against bacitracin, methicillin, novobiocin, lomefloxacin, kanamycin, amikacin, ciprofloxacin and spectinomycin.

Key words: EPEC; HUS; STEC; TTP.

Shiga-toxin producing (STEC) and enteropathogenic *Escherichia coli* (EPEC) are serologically diverse group of emerging food borne pathogens. These are responsible for causing a spectrum of illness in humans ranging from haemorrhagic diarrhoea to even fatal consequences such as hemolytic uraemic syndrome (HUS), thrombotic thrombocytopenic purpurea (TTP) and haemorrhagic colitis (HC) (Croxen and Finlay, 2010; Gyles and Fairbrother, 2010). STEC strains produce one or both of two major types of Shiga toxin, designated Stx_1 and Stx_2. The production of the Stx_2 is associated with an increased risk of developing HUS (Boerlin *et al.*, 1999). The central mechanism of EPEC pathogenesis is a lesion called 'attaching and effacing' (A/E), which is characterized by adherence of bacteria to the intestinal epithelium. EPEC strains are a major cause of infantile diarrhoea in developing and developed countries and are responsible for thousands of death worldwide (Ochoa *et al*. 2008). They are also associated with diarrhea in most domestic animals species (Bardiau *et al*., 2010). Enteropathogenic *E. coli* have also been associated with diarrhoea in pigs (Vu- Khac *et al*., 2007). These attaching and effacing *E. coli* possess the *eae* gene encoding the outer membrane protein known as intimin, involved in attachment of the bacteria to gastroinestinal epithelial cells (Vu- Khac *et al*., 2007).

Taking into account that there is a paucity of literature on the prevalence status of STEC and EPEC infection among pigs in India, the present

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study was undertaken to isolate and characterize shiga toxin producing (STEC) and enteropathogenic *E. coli* from piglets in Mizoram state, India.

MATERIALS AND METHODS

Sampling and isolation of *E. coli*

During this study, a total of 100 fecal samples originating from 40 healthy and 60 diarrhoeic piglets (0–3 month) were collected from different parts of Mizoram state. The samples were collected directly from rectum using sterile swabs and processed immediately for isolation of *E. coli*. The samples were inoculated directly on Mac Conkey Agar and five characteristic colonies were randomly selected and subcultured on eosin methylene blue (EMB) agar. Later, a well separated presumptive *E. coli* single colony was picked up on nutrient agar slants as pure culture and subjected to standard morphological and biochemical testing as described by Ewing (1986) to confirm their identity.

Serotyping

E. coli isolates obtained in this study got serotyped from National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli H.P. 173204 (India).

Templates DNA preparation

The *E.coli* isolates confirmed by conventional tests were grown in Luria Bertani broth at 37ºC overnight. One ml of the broth culture was pelleted by centrifugation at 8000 rpm for 10 minutes, washed twice with 500µl of PBS (pH 7.4). The bacterial pellet was finally, re-suspended in 300µl sterile nuclease free water and lysed by boiling for 10 minutes in a water bath followed by immediate chilling for 10 minutes on ice. The lysates were centrifuged again at 6000 rpm for 10 minutes and the supernatant was used as template DNA.

Detection of virulence genes by multiplex PCR

Multiplex PCR based screening of stx_1, stx_2, *eae*A and *hly*A virulence genes (Table 1) among the isolates was carried out as described by Paton and Paton (1998). Amplified products were analysed by agarose gel (2%) electrophoresis and documented.

Antibiogram

In vitro antimicrobial susceptibility pattern of the STEC and EPEC isolates obtained in the present study was determined by disc diffusion method (Bauer *et al*., 1966) using 16 commonly used antimicrobial agents. The antimicrobial discs (HiMedia) used were: Amoxycillin (30 mcg), Amikacin (30 mcg), Bacitracin (10 units), Cefazolin (30 mcg), Cefixime (5 mcg), Chloramphenicol (30 mcg), Ciprofloxacin (5 mcg), Gatifloxacin (5 mcg), Gentamicin (10 mcg), Kanamycin (30 mcg), Lomefloxacin (10 mcg), Methicillin (10 mcg), Novobiocin (30 mcg), Oflaxacin (5 mcg), Spectinomycin (100 mcg) and Tobramycin (10 mcg). The results were noted after 24 h of incubation at 37°C by measuring the diameter of zone of inhibition and interpretation as sensitive, intermediate or resistant were made as per manufacturer's instructions.

RESULTS

Out of the 500 presumptive *E. coli* isolates picked from 100 samples, 254 isolates were confirmed by biochemical tests of which 132 were from piglets with diarrhoea (60) and 122 from non diarrhoeic piglets (40). In Serotyping, a total of 22 different serogroups have been recorded with 90 remained as untypeable. In Multiplex PCR based screening of virulence gene showed that out of 254 isolates, 51 (20.08%) were carried at least one virulence gene, based on which eight different virulence gene profile were identified. Further, based on the prevalence of virulence genes, these 51 isolates could be classed into 30 (11.81%) and 21 (8.26%) STEC and EPEC isolates, respectively. All the 51 strains were classified under 8 genotypic profiles (Table 3) where 2 (0.78%), 9 (3.54%), 2 (0.78%), 7 (2.75%), 1 (0.39%), 9 (3.54%), 4 (1.57%), 17 (6.69%) were carried stx_1 only, stx_2 only, stx_1 and stx_2, stx_2 and *eae*A, stx_2 and *hly*A, stx_2, *eae*A and *hly*A, stx_2, *eae*A and *hly*A, *eae* and *hly*A respectively. None of the strains showed *hly*A gene only. All the 26 (19.69%) virulence gene positive strains from diarrhoeic samples were categorized into 6 genotypes whereas all the 25 (20.49%) virulence gene strains from healthy samples were categorized into 8 different genotypes (Table 2).

In this study, a sizeable number of STEC and EPEC strains expressed the resistance to more than one antimicrobial agents (Table 3). All the STEC strains exhibited 100% resistance

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

S. No.	Primer	Sequence	Amplicon size	Reference
1	stx1F	5'-ATAAATCGCCATTCGTTGACTAC-3'	180bp	Paton and Paton, 1998
	stx1R	5'-AGAACGCCCACTGAGATCATC-3'		
2	stx2F	5'-GGCACTGTCTGAAACTGCTCC-3'	255bp	Paton and
	stx2R	5'-TCGCCAGTTATCTGACATTCTG-3'		Paton, 1998
3	eaeAF	5'-GACCCGGCACAAGCATAAGC-3'	384bp	Paton and Paton, 1998
	eaeAR	5'-CCACCTGCAGCAACAAGAGG-3'		
4	hlyAF	5'-GCATCATCAAGCGTACGTTCC-3'	534bp	
	hlyAR	5'-AATGAGCCAAGCTGGTTAAGCT-3'		Paton and Paton, 1998

Table 2. Distribution of virulence genes in *E . coli* from piglets with or without diarrhoea in Mizoram

S.No	Virulence	Diarrhoeic	Non diarrhoeic	Total
1	stx_1 only	1 (O24)	1 (O172)	2 (0.78%)
2	stx_2 only	6 (O75, O119, UT)	3 (O24, 0120)	9 (3.54%)
3	stx_1 and stx_2	-	2 (O119, O161)	2 (0.78%)
4	stx_2 and *eae*A	3 (O2, UT)	4 (O51, O97, O132, O142)	7 (2.75%)
5	stx_2 and *hly*A	-	1 (O24)	1 (0.39%)
6	stx_2, *eae*A and *hly*A	5 (O75, O119, O159, O165)	4 (O2, O69,UT)	9 (3.54%)
7	*eae*A only	2 (O60, O75)	2 (O141, UT)	4 (1.57%)
8	*eae*A and *hly*A	9 (O103,O119, O132, O162, O163)	8 (O56, O60, O119,O168)	17 (6.69%)
9	Total	26 (19.69%)	25 (20.49%)	51 (20.08%)
10	STEC	15 (11.36%)	15 (12.29%)	30 (11.81%)
11	EPEC	11 (8.33%)	10 (8.19%)	21 (8.26%)

Table 3. Antibiograms of STEC and EPEC isolated from piglets with or without diarrhoea. Figures in parenthesis are indicating the percentage

S.	Antimicrobial	Concentration	STEC Isolates	EPEC Isolates		
No	agents	per discs	Sensitive (n=30)	Resistance (n=30)	Sensitive (n= 21)	Resistance (n= 21)
1	Amoxycillin (Am)	30 mcg	27 (90%)	3 (10%)	18 (86%)	3 (14%)
2	Amikacin (Ak)	30 mcg	18 (60%)	4 (40%)	12 (58%)	9 (42%)
3	Bacitracin (B)	10 units	nil	30 (100%)	nil	21 (100%)
4	Cefazolin (Cz)	30 mcg	29 (80%)	6 (20%)	20 (95%)	1 (5%)
5	Cefixime (Cfx)	5 mcg	27 (90%)	3 (10%)	19 (90%)	2 (5%)
6	Chloramphenicol (C)	30 mcg	21 (70%)	9 (30%)	16 (78%)	5 (22%)
7	Ciprofloxacin (Cf)	5 mcg	18 (60%)	12 (40%)	19 (90%)	2 (10%)
8	Gatifloxacin (Gf)	5 mcg	27 (90%)	3 (10%)	19 (90%)	2 (10%)
9	Gentamicin (G)	10 mcg	24 (80%)	6 (20%)	21 (100%)	Nil
10	Kanamycin (K)	30 mcg	15 (50%)	15 (50%)	7 (33%)	14 (67%)
11	Lomefloxacin (Lo)	10 mcg	3 (10%)	27 (90%)	10 (48%)	11(52%)
12	Methicillin (M)	5 mcg	nil	30 (100%)	nil	21 (100%)
13	Novobiocin (Nv)	30 mcg	nil	30 (100%)	nil	21 (100%)
14	Oflaxacin (O)	5 mcg	21 (70%)	9 (30%)	21 (100%)	Nil
15	Spectinomycin (Se)	100 mcg	21 (70%)	9 (30%)	5 (25%)	16 (75%)
16	Tobramycin (Tb)	10 mcg	21 (70%)	9 (30%)	19 (90%)	2 (10%)

against bacitracin, methicillin and novobiocin whereas 90% strains showed the resistance against lomefloxacin. None of the STEC isolates were found to be 100% sensitive against any of the antimicrobial agents studied. Similarly, in case of EPEC 100% resistance were recorded against bacitracin, methicillin and novobiocin, but in contrast to STEC 100% sensitivity of all the EPEC strains were recorded against gentamicin and lomefloxacin.

DISCUSSION

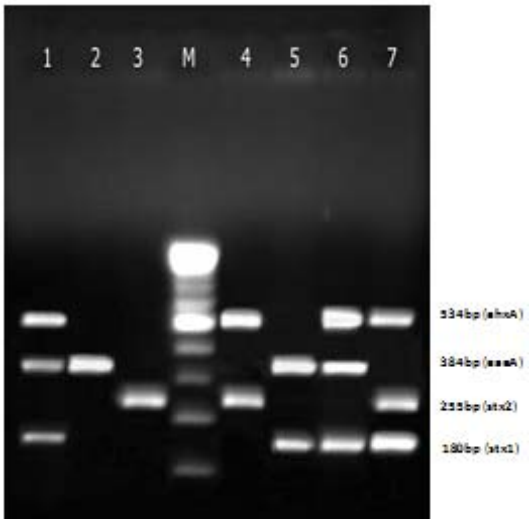

Fig. 1. Multiplex PCR based molecular characterization of Shiga-toxigenic *E. coli* and enteropathogenic *E. coli* isolated from piglets with or without diarrhea. Lane-M: 100 bp DNA ladder. Lane-1, 2, 3, 4, 5, 6 & 7 are representing different virulence gene(s) of STEC/EPEC

Perusal literature regarding the STEC infection in India shows reports are available for cattle (Pal *et al*., 1999), sheep (Wani *et al*., 2004; Bhat *et al*., 2008), fish (Kumar *et al*., 2001), beef (Khan *et al*., 2002), pigs (Barman *et al*., 2008), Yaks (Bandhopadhyay *et al*., 2009), poultry (Dutta *et al*., 2011) and human faeces (Chattopadhya *et al*., 2001).

Schierack *et al*. (2006) reported that porcine pathogenic *E. coli* strains belonged to a limited number of serogroups *viz.* 08, 0108, O138, O139, O141, O149 and O157. Barman *et al*. (2008) also reported that most of the edema disease causing STEC in pigs belongs to the serogroup O138, O139, O141 and O147 including few untypable strains. On the contrary, the serotypes identified in this study belonged to different somatic antigen types which were not reported by earlier workers in India. Among the 254 isolates, a total of 22 different serotypes were identified, with O119 (44) being the most predominant serotype identified. As several new serogroup of *E. coli* are being established as potential pathogens for human and animals, the clinical significance of O119 serotype in piglet diarrhoea needs to explored by further studies.

The virulence gene profile of the STEC isolates showed that the prevalence of stx_2 gene was higher than stx_1 gene which was in agreement to the findings of earlier workers (Bandyopadhyay *et al*., 2012 and Kim *et al*., 2010). The stx_2 gene is considered to be the most important virulence factor which in mice was shown to be 400 fold more toxic than stx_1 and it also induces feto-placental re-absorption, intrauterine hematoma, fibrin deposition and neutrophil infiltration (Tesh *et al*., 1993). Thus isolation of STEC isolates with high frequency of stx_2-positivity from piglets is a serious concern as strains expressing stx_2 are more likely to be associated with the development of HUS (Islam *et al*., 2007; Pradel *et al*., 2008). It also indicates the magnitude of virulence, of the *E. coli* strains circulating among pigs in this region. Infection of humans with these virulent *E. coli* strains could have serious public health consequences. Also in this study, only 2 (3.92%) of STEC isolates was found to carry both stx_1 and stx_2 genes which is much lower compared to earlier reports of Bhat *et al*. (2008) where in 41.71% STEC isolates obtained from lambs carried both stx_1 and stx_2 genes. Out of 21 EPEC strains, 17 (80.95%) isolates carried both *eae*A and *hly*A gene and 4 (19.05%) carried *eae*A gene alone. The major pathogenicity of EPEC was reported to be mediated by the products of locus of enterocyte effacement (LEE) pathogenicity island with the *eae*A gene encoding for intimin protein involved in the intimate adhesion of bacteria to enterocytes and production of attaching and effacing (AE) lesions on the intestinal mucosa (Paton and Paton, 1998). Therefore, the prevalence of such kind of *E. coli* strains might result in increased pathogenicity for pigs.

The antimicrobial sensitivity pattern of

the STEC and EPEC isolates obtained in the present study revealed that these are significantly resistant against bacitracin, methicillin and novobiocin. None of the STEC isolates was found to be 100% sensitive against any of the antimicrobial agents studied but all the EPEC strains were found to be sensitive to gentamicin and lomefloxacin. Schroeder *et al*. (2002) reported the highest prevalence of antimicrobial resistance among swine O157 isolates. Similar findings were also reported by Umera *et al*. (2003), Choi *et al*. (2002) and Bandopadhyay *et al*. (2012). Multiple antimicrobial resistance in STEC and EPEC may results from the spread of genetic elements including plamids, transposons, integrons (Zhao *et al*., 2001). The transfer of antibiotic resistance genes are often related to integrons, which are capable of capturing and inserting antibiotic resistance genes into their structure (Hall and Stokes, 1993). Since antimicrobial resistant bacteria from food animals may colonize the human population via-food chain, contact through occupational exposure or waste run off from animal production facilities (Van den Bogaard and Stobberieng, 1999), it is possible that these resistant bacteria could readily be transferred from food animals to humans (Schroeder *et al*., 2002). The emergence of multiple antimicrobial resistance may complicate future therapeutic options. Thus prevalence of multiple drug resistant STEC and EPEC isolates in high proportion among piglets in this state indicates that these animals act as an important reservoir which poses serious threat to a public health.

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