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Detection and characterization of shiga toxigenic *E. coli* (STEC) and enteropathogenic *Escherichia coli* (EPEC) from diarrhoeic piglets in an organized farm in Kolkata

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ABSTRACT

Escherichia coli were isolated from fecal samples of diarrhoeic piglets from an organized farm in Kolkata, West Bengal, India. All the *E. coli* strains (n=65) were characterized morphologically and antibiotic sensitivity assay. Virulence genes (*stx*$_1$, *stx*$_2$, *eaeA* and *hlyA*) were detected by multiplex PCR assay. Sixty-five *E. coli* strains obtained from 35 fecal samples. Altogether, 6 serogroups were recorded, and O20 was the most prevalent. Out of 65 *E coli* strains, 6 (9.32%) carried at least one virulence gene of which 5 and 1 were identified as STEC and EPEC, respectively. Majority of the isolates were multi-drug resistant. Association of STEC and EPEC with diarrhoea in piglets indicates that these animal species represent as an important reservoir of these organisms for human infection in this part of the globe. Detection of multi-drug resistance STEC and EPEC with virulence genes in piglets also indicates the importance of this organism as a potential zoonotic threat to human population in this region.

Key words: EPEC, Piglets, STEC, Virulence genes

The morbidity and mortality associated with several recent large outbreaks of gastrointestinal diseases caused by shiga toxin-producing *Escherichia coli* (STEC) indicate the threat these organisms pose to public health (Paton and Paton 1998). STEC are commonly recovered from food producing animals and pose a serious threat to humans and other livestock (Narato and Kaper 1998). STEC causes a serious gastrointestinal and systemic diseases such as haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) leading to diarrhoea, especially among the infants in the developing countries (Paton and Paton 1998). STEC strains produce 1 or both of the 2 major types of shiga toxins, designated *Stx*$_1$ and *Stx*$_2$, and the production of the later is associated with an increased risk of developing HUS (Boerlin *et al*. 1999).

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, only few reports are available on isolation, identification and characterization of STEC in human and animals (Pal *et al*. 1999, Chattopahdhaya *et al*. 2001, Kumar *et al*. 2001, Khan *et al*. 2002, Wani *et al*. 2004, Bhat *et al*. 2008). In the present study, we have investigated the association of STEC/EPEC as a probable cause of piglet diarrhoea in Kolkata, West Bengal, India.

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MATERIALS AND METHODS

Animals and sampling: In an organized farm in Kolkata, West Bengal, 230 piglets were present along with 100 sows, 40 gilt and 12 boars. Rectal swabs collected from all the 35 piglets showed diarrhoea. Samples were collected by sterile cotton swabs and transported to the laboratory under cold chain for further bacteriological examination.

Isolation of E. coli *from clinical specimens:* After reaching to the laboratory, samples were immediately inoculated on MacConkey's agar plate and incubated at 37°C for 18–24 h. After incubation, 5 randomly selected lactose fermenting colonies were picked up and subcultured on eosin methylene blue (EMB) agar plates to observe the characteristic metallic sheen of *E. coli*. The well separated pure colonies were picked up on nutrient agar slants as pure culture and subjected for standard morphological and biochemical tests (Edwards and Ewing 1972). From 35 clinical samples 65 *E. coli* strains were isolated.

Serotyping of E. coli*:* The 65 *E. coli* strains were serotyped

Table 1. Oligonucleotide primers used in multiplex PCR assay for detection of virulence genes

Primers	Primer sequences	References
stx$_1$ F	5´-ATA AAT CGC CAT TCG TTG ACT AC-3´	Paton and Paton (1998)
stx$_1$ R	5´- AGA ACG CCC ACT GAG ATC ATC -3´	
stx$_2$ F	5´- GGC ACT GTC TGA AAC TGC TCC -3´	Paton and Paton (1998)
stx$_2$ R	5´- TCG CCA GTT ATC TGA CAT TCT G -3´	
eaeA F	5´- GAC CCG GCA CAA GCA TAA GC -3´	Paton and Paton (1998)
eaeA R	5´- CCA CCT GCA GCA ACA AGA GG -3´	
ehxA F	5´- GCA TCA TCA AGC GTA CGT TCC -3´	
ehxA R	5´-AAT GAG CCA AGC TGG TTA AGC T-3´	

Source: Paton and Paton (1998).

based on their somatic (O) antigens at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, Himachal Pradesh.

Antibiogram of E. coli*:* All the strains were subjected to *in vitro* antibiotic sensitivity test as per Kirby Bauer disc diffusion method (Bauer *et al*. 1966) using 18 commonly used antibacterial agents (Table 2).

Preparation of E. coli *DNA for PCR assay:* For rapid detection of virulence genes, isolated bacterial cultures were inoculated into 2 ml Luria Bertani (LB) broth and incubated at 37°C under constant shaking for 18 h. After incubation, 1 ml broth was taken in a 1.5 ml microcentrifuge tube and centrifuged at 8000 rpm for 10 min. The pellet was washed twice in sterile NSS and resuspended in 400 µl of nuclease free sterile distilled water and boiled for 10 min followed by immediate chilling. Cell debris was removed by centrifugation at 5000 rpm for 5 min. The supernatant was used as template DNA for the PCR reaction.

Table 2. Antimicrobial sensitivity/resistance pattern of *E. coli* isolates from diarrhoeic piglets against 18 commonly used antibacterial agents

Antimicrobial agents	Concentration	Strains sensitive (n=65)	Strains resistance (n= 65)
Amoxycillin (Am)	10 µg	53 (81.54)	12 (18.46)
Ampicillin (A)	10 µg	20 (30.77)	45 (72.31)
Cefixime (Cfx)	5 µg	24 (36.92)	41 (63.08)
Cephotaxime (Ce)	10 µg	55 (84.62)	10 (15.38)
Chloramphenical (C)	10 µg	49 (75.38)	16 (26.62)
Chlortetracycline (Ct)	30 µg	61 (93.85)	4 (6.15)
Ciprofloxacin (Cf)	10 µg	55 (84.62)	10 (15.38)
Erythromycin (E)	10 µg	19 (29.23)	46 (70.77)
Enrofloxacin (Ex)	10 µg	62 (95.38)	3 (4.62)
Gentamicin (G)	10 µg	26 (40.00)	39 (60.00)
Lincomycin (L)	10 µg	14 (21.54)	51 (78.46)
Nalidixic acid (Na)	30 µg	13 (20.00)	52 (80.00)
Oxytetracycline (O)	30 µg	15 (23.08)	50 (76.92)
Penicillin (P)	10 µg	21 (32.31)	44 (67.69)
Roxythromycin (Ro)	30 µg	25 (38.46)	40 (61.54)
Streptomycin (S)	10 µg	27 (41.54)	38 (58.46)
Sulfadiazine (Sz)	100 µg	23 (35.38)	42 (64.62)
Tetracycline (T)	10 µg	22 (33.85)	43 (66.15)

Figures in parenthesis are indicating the percentage

Detection of virulence genes by multiplex PCR: A multiplex PCR was carried out using 4 sets of oligonucleotide primers for *stx*$_1$, *stx*$_2$, *eaeA* and *hlyA* genes. The primers used in this study are given in Table 1. The multiplex PCR mixture of 25 µl contained 1X PCR buffer, 1.5 mM of $MgCl_2$, each primer within the 4 primer sets at a concentration of 40 nM, 200 µM each of dNTPs, 1.0 U of *Taq* DNA polymerase and 2.0 µl of template DNA. The PCR reaction was performed in a thermal cycler using the following standard cycling procedure: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45s, primer annealing at 59°C for 45s and extension at 72°C for 1 min and a final extension at 72°C for 6 min.

Amplified products were separated by agarose gel (2% in 1X Tris-borate-EDTA buffer) electrophoresis at 5v/cm for 2 h and stained with ethidium bromide (0.5 µg/ml). Standard molecular size marker (100 bp DNA ladder) was included in each gel. DNA fragments were observed by ultraviolet transilluminator and photographed in a gel documentation system.

The PCR reaction was performed 3 times to ensure the repeatability of the techniques and to make sure that strains were correctly assigned to respective patterns.

RESULTS AND DISCUSSION

Bacterial isolation and characterization: A total of 65 *E. coli* strains were isolated from 35 fecal samples. All the 65 *E. coli* strains belonged to 64 serogroups, viz. O20 (15), O138 (14), O172 (10), O51 (9), O60 (8) and 5 of them were untypable (UT).

Antimicrobial sensitivity test: The result of antimicrobial sensitivity patterns of *E. coli* strains against 18 commonly used antimicrobial agents are presented in Table 2. Majority of the strains exhibited high level of resistance to nalidixic acid (80.00%), lincomycin (78.46%), oxytetracyclin (76.92%), ampicillin (72.31%), erythromycin (70.77%), penicillin (67.69%), tetracycline (66.15%), sulfadiazine (64.62%), cefixime (63.08%), roxythromycin (61.54%),

Table 3. Virulence genes profile of *E. coli* strains isolated from diarrhoeic piglets from an organized farm in Kolkata, West Bengal

Serogroup	No of isolates	Stx_1	Stx_2	*eaeA*	*hlyA*
O138	4	+	+	–	–
O60	1	+	+	–	+
O138	1	–	–	+	–
Total	6	5	5	1	1

gentamicin (60.00%), and streptomycin (58.46%). The best antimicrobial agent in terms of sensitivity was enrofloxacin (95.38%) followed by chlortetracycline (93.85%), cephotaxime (84.62%), ciprofloxacin (84.62%), amoxycillin (81.54%) and chloramphenical (75.38%).

Multiplex PCR for virulence genes: Multiplex PCR assay gave amplified products of ~180 bp, ~255 bp, ~384 bp and ~534 bp specific for stx_1, stx_2 and *eaeA* and *hlyA* genes, respectively.

Out of 42 *E. coli* strains from poultry with diarrhoea, 6 (9.23%) carried at least 1 virulence gene studied, out of which 5 (7.69%) and 1 (1.54%) strains were detected as STEC and EPEC, respectively. Out of 5 STEC strains 1 carried stx_1, stx_2, and *hlyA* genes and 4 carried stx_1 and stx_2 genes. The lone EPEC strain carried *eaeA* gene only (Table 3).

In India information regarding the STEC is available on cattle (Pal *et al*. 1999), sheep (Wani *et al*. 2004, Bhat *et al*. 2008), fish (Kumar *et al*. 2001) as well as on beef (Khan *et al*. 2002) and human feces (Chattopahdhaya *et al*. 2001, Khan *et al*. 2002). But information related with prevalence of STEC and EPEC in piglets is rare. In Eastern India, particularly the metropolitan city Kolkata, pork is one of the major sources of animal protein to the people. In this study, majority of the *E. coli* strains expressed the resistance to more than one antimicrobial agent (Table 2). Schroeder *et al.* (2002) reported the highest prevalence of antimicrobial resistance where greater than 50% of all isolates were resistance to sulfamethoxazole, cephalothin, tetracycline and more than 20% isolates were resistant to ampicillin and gentamicin. Similar observations were also reported from Japan (Uemura *et al*. 2003) and Korea (Choi *et al*. 2002). Multiple antimicrobial resistances in STEC and non-STEC may partly result from the spread of genetic elements including plasmids, transposons and integrons (Zhao *et al*. 2001). Because antimicrobial resistant bacteria from food animals may colonize the human population via food chain, contact through occupational exposure or waste runoff from animal production facilities (Van den Bogaard and Stobberingh 1999), it is possible that resistant bacteria may be readily transferred from food animals to humans (Schroeder *et al.* 2002).

In this study 6 different serogroups were recorded amongst the 65 *E. coli* strains of which O20 (23.08%) is the predominant followed by O138 (21.54%), O172 (15.38%), O151 (13.85%), O60 (12.31%) and O5 (6.15%). Schierack *et al.*(2006) reported that porcine pathogenic *E. coli* strains belong to the limited number of serogroups, with O8, O108, O138, O139, O141, O149 and O157. Barman *et al.* (2008) also reported that most of the oedema causing STEC in pigs belongs to the serogroups O138, O139, O141 and O147 as well as few untypable strains. In our study, O138 was isolated from diarrhoeic piglets. But isolation of high level of O20 serogroup *E. coli* from diarrhoeic piglets warrants a special attention to study about their role in development of piglet diarrhoea. As a lot of new serogroups are being established as potential pathogens for both human and animals, therefore getting new serogroup in the form of O20 cannot be overlooked as non-pathogenic strains. This is indicating that pigs in this region are acting as a potential reservoir of pathogenic *E. coli*, which may cause acute outbreak at any point of time. Once the organism is in the pig's environment, it is difficult to eliminate and can be carried to other areas on the farm or off the farm to other swine herds (Helgerson *et al*. 2006).

Out of 65 *E. coli* strains tested in this study, 6(9.23%) carried at least one virulence gene studied, out of which 5 (7.69%) and 1 (1.54%) strains were detected as STEC and EPEC, respectively. Prevalence of stx_1 and stx_2 genes was similar, which is not in agreement with the findings of other investigators in India and abroad, who mentioned the higher incidence of stx_1 genes than stx_2 gene in STEC from animals in India (Bhat *et al*. 2008), Australia (Djordjevic *et al*. 2001) and Spain (Orden *et al*. 2003). This type of variance may be because of small sample size under the present investigation.

In this study, only 1 (1.44%) EPEC isolate carried only *eaeA* gene. The major pathogenicity of EPEC is mediated by the products of the locus of enterocyte effacement (LEE) Pathogenicity Island, with the *eae* gene that encodes for the intimin protein involved in the intimate adhesion of bacteria to enterocytes and production of AE lesion on the intestinal mucosa (Paton and Paton 1998). Besides, a specific plasmid encoded haemolysin called EHEC haemolysin, which is encoded by *hlyA* gene, might contribute to the virulence of STEC in humans (Beutin *et al*. 1995). Therefore, presence of such kind of *E. coli* in pig's environment might increase the possibility of infection to the human beings. Presence of even low level of *eae* gene in STEC isolates should not be overlooked because some *eae*-negative STEC were also isolated from human beings with clinical symptoms (Strockbine *et al*. 1997, Kumar *et al*. 2004).

STEC/EPEC is associated with piglet diarrhoea, and high presence of these organisms in the healthy and diarrhoeic piglets indicates that these animal species represent as an important reservoir of these organisms for human infection in this part of the globe. Detection of multi-drug resistance STEC/EPEC with virulence genes in piglets also indicates the importance this organisms as a potential zoonotic threat to human population in this region.

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