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Biochemical and Molecular Detection of Enteropathogenic *Escherichia coli* (EPEC) from Human and Porcine Diarrheic Cases in Assam, India

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ABSTRACT

In North Eastern region of India pig rearing is an important livestock farming occupation with cohesive habitation of humans and pigs. A variety of diarrhoeal diseases in these two hosts occur due to pathogenic forms of *E. coli* harbouring virulence, specific colonisation factors and pathogenicity associated genes. 115 faecal samples were collected from human and pigsin Assam, India out of which 93 samples were positive for *E. coli* presence resulting in 80.80 percent positivity for isolation. Out of these, 51 were positive for human and 42 were positive for pig. The isolated *E. coli* confirmed by morphological and biochemical identification. Molecular characterisation studies targeting the virulence genes viz., stx_1, stx_2, *eae* for shiga toxin producing *E. coli* (STEC) was made by multiplex PCR using a cocktail of type specific primers. The presence of enteropathogenic *E. coli* (EPEC) among the positive isolates was identified by simplex PCR aiming the *eae* and *bfp* genes. Among the 51 human isolates, 20 were EPEC and none contained the *bfp* gene, signifying as atypical EPEC. However, none of the pig isolates were found to be typable. This baseline study on pathogenic form of *E. coli* may help in further serogrouping of the organism for diagnostics and pathogenesis implications.

Keywords: Enteropathogenic, *E. coli*, shiga toxin, multiplex PCR, Assam

Escherichia coli are usually considered as commensal organism; however, some strains have the capacity to cause disease in human and animals. These disease causing strains are called diarrheagenic which include pathogens having public health importance worldwide and are further divided into six categories depending on their virulence factor (Nataro and Kaper, 1998). Enterotoxigenic *E. coli* (ETEC) are the most commonly reported diarrheagenic *E. coli* strains and it produce one or more enterotoxins which are heat labile LT (LT-1 and LT-2) or heat stable ST (STa and STb) (Levine, 1987). Shiga toxin producing *E. coli* (STEC) is categorised as such due to its ability to produce two potent cytotoxin, viz., Shiga-like toxins 1 and 2 (Stx1 and Stx_2) (Tesh, 1998) whereas in some strains LEE locus is present which is associated to the attaching and effacement lesion (Jarvis and Kaper, 1996; Stacy *et al.*, 1995). Another group namely, enteropathogenic *E. coli* (EPEC) do not produce any classic toxins. They induce virulence by producing attaching and effacement lesions of the intestinal microvilli of the host cell (Jerse *et al.,* 1990). A cluster of genes encoding bundle forming pili (*bfp*) is present on the large EPEC adherence factor (EAF) plasmid (Kaper, 1996). All EPEC strains do not possess EAF plasmid, hence, those that do are termed as typical EPEC and those that do not are called atypical EPEC (Trabulsi *et al.,* 2002). Enteroinvasive *E. coli* (EIEC) conquer the epithelial cells of the colon, where it multiplies, resulting in necrosis of the tissue. It is often mistaken with *Shigella* due to its biochemical, physiological and genetic similarities with the latter. A virulence plasmid (pInv) of 140 MDa that encodes a type III secretion system harbour the genes related to invasion (Berhutti *et al.,* 1998; Nataro, 2002). Enteroagrregative *E. coli* (EAggEC) strains are characterized by their ability to aggregately adhere to

tissue culture cells. Fimbrial structures, namely, AA fimbriae I and II are associated with adherence to Hep-2 cells and human erythrocytes (Czeczulin *et al.,* 1997) and they are encoded by *aggR* genes. Diffuse adherent E. coli (DAEC) strains have the ability to adhere to Hep-2 cells in a non-localized manner. A surface fimbria (F1845) has been suggested as a putative virulence factor that could be facilitating this adherence phenotype (Bilge *et al.,* 1989).

Apart from humans, pathogenic *E. coli* is also responsible for a variety of intestinal disorders such as diarrhoea and edema disease syndrome in pigs (Martins *et al.*, 2000 and Vu-Khac*et al.,* 2007). In neonatal and recently weaned pigs, enterotoxigenic *E. coli* (ETEC) is a major cause of illness and death.

Thus, the present study aimed at isolation of *E. coli* from diarrheic as well as non-diarrheic faeces of human and pig which dwelled in a common habitat from different pig rearing households of Assam, India. Further, the study emphasized on molecular detection of toxigenic strains infecting the human and pig population with a purpose to look for if there is any common toxin strains circulating between the two hosts.

MATERIALS AND METHODS

Collection of samples

A total of 115 diarrhoeic faecal samples were collected from human and pigs from different parts of Assam, India. Out of these, 62 were samples collected from human from hospitals and dispensaries with acute cases of diarrhoea and also from households dwelling in close proximity with pigs and the remaining 53 were pig faecal samples collected from households and unorganised pig farms. The samples were collected in sterile screw capped container and brought to the laboratory for processing and isolation of *Escherichia coli.*

Isolation of *Escherichia coli*

For isolation of *E. coli*, samples were inoculated in brain heart infusion (BHI) broth (Chapman, 1946) and incubated aerobically for 24 hrs at 37ºC. Turbidity was observed in the cultures and thereafter subculture was done on eosin methylene blue (EMB) agar (American Public Health association, 1950) as well as on MacConkey Lactose agar plates for colony identification and morphological study. Microorganisms which grow on MLA are capable of metabolizing lactose which in turn produces acid by-products that lowers the pH of the media and causes the neutral red indicator to turn red (Koneman, 2005).

Morphological identification and biochemical detection

Suspected colonies of *E. coli* producing metallic sheen on EMB agar plates were further confirmed by Gram's staining as per earlier described protocol (Edwards and Ewing, 1986). For biochemical detection, colonies were sub cultured in BHI broth and incubated for 24hrs at 37ºC and then, centrifuged at 13000 rpm for 10 minutes and the supernatant was collected. Different biochemical tests (Werkman, 1930; O'Meara, 1931; Vaughn *et al.*, 1939 and Silva, 1980) were performed for identification of *E. coli* isolates by using Hi24 *Enterobacteriaceae* identification kit from Himedia.

Antibiotic sensitivity test

For antibiotic sensitivity test, a single colony of *E. coli* isolate was picked and inoculated in BHI broth and incubated for 24hrs at 37ºC. Now, a sterile cotton swab was taken and dipped into the *E. coli* containing broth and spread evenly on sterile nutrient agar (NA) plate. The plates were allowed to dry for 5 minutes. Using applicator, antibiotic discs were placed onto the surface of NA plates and gently pressed and incubated at 37ºC for 24 hrs. For disc diffusion testing, following antibiotics were used: Ciprofloxacin (5 mcg), Chloramphenicol (30 mcg), Ofloxacin (5 mcg), Ampicillin (10 mcg), Erythromycin (15mcg), Levofloxacin (5 mcg), and Streptomycin (10 mcg) from Himedia Laboratories. The inhibition zone diameters were measured by slipping callipers and compared with standard strain *E. coli* ATCC 25922 from the zone interpretative chart (Himedia) (Bauer *et al.*, 1966).

DNA extraction

DNA was extracted from each bacterial culture by mixing 3-4 pure colonies directly into sterile miliQ water. The suspensions was then boiled for 10 minutes and snap chilled on ice for 20 minutes (hot cold lysis) and centrifuged at

12,000 rpm for 10 minutes. The supernatant containing DNA was collected and the concentration of extracted DNA was assessed by Nano drop spectrophotometer (Thermo Scientific, USA).

Molecular detection of *stx1*, *stx2*, *eae* and *bfp* gene by multiplex PCR

To reveal the pathogenicity of all the *E. coli* isolates confirmed by morphological and biochemical detection, they were subjected to a multiplex PCR assay targeting three virulence genes namely, stx_1, stx_2, *eae* and *bfp* as earlier described protocol (Vidal, 2005) using a cocktail of specific primers. The details of primers are shown in Table 1.

RESULTS AND DISCUSSION

In the present study, a total of 115 diarrhoeic faecal samples were collected from human and pigs from different parts of Assam.

Isolation, morphological identification and biochemical detection of *E. coli*

E. coli was isolated on EMB agar plate and MLA plate for morphological detection. Culture on EMB agar plates showed green metallic sheen after 24 hours and pink colonies on MLA plates. Based on colony morphology and Gram's staining, 93 samples were found to be positive out of 115, which resulted in 80.8 per cent positivity. In case of humans, 51 were positive out of 62 (82.2%) and in case of pigs 42 were positive out of 53 (79.2) as shown in Table 2. EMBA and MLA plates are shown in Fig. 1 (a) and (b). Gram's stain result is shown in Fig. 2.

Table 2: No. of samples collected and positive for *E. coli* from different sources

Sources	Nature of Faecal Samples	Number of samples collected	No. of positive samples	Per cent positivity
Human	Diarrhoeic	62	51	82.2
Pig	Diarrhoeic	53	42	79.2
Total		**115**	**93**	**80.8**

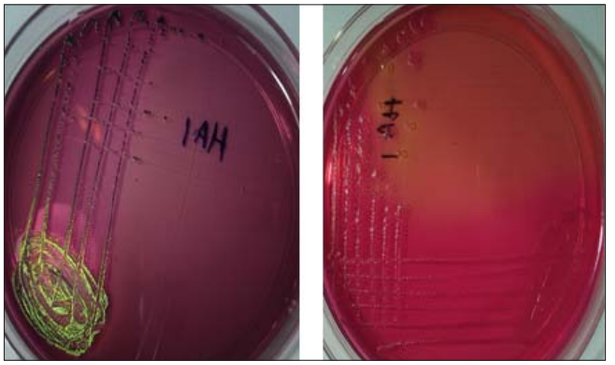

Fig. 1: (a) EMB agar plate showing green metallic sheen producing pure colony of *E. coli* **(b)** MLA plate showing lactose fermentation of *E. coli*

The present study relates with a previous study (Borah, 1994) where *E. coli* was isolated from 86.11 % of pigs. The present finding is also quite close with the results of a previous finding (Do Tn *et al.,* 2006) from North Vietnam who reported to find diarrhoea in 71.5% of the litter born

Table 1: List of primer sequences and amplicon size

Sl. No.	Oligo Name	Gene	Primer	Sequence (5' → 3')	Oligo Length	Amplicon Size (bp)
1	ES1F	stx_1	Forward	CAGTTAATGTGGTGGCGAAGG	21	348
2	Es1R		Reverse	CACCAGACAATGTAACCGCTG	21	
3	ES2F	stx_2	Forward	ATCCTATTCCCGGGAGTTTACG	22	584
4	ES2R		Reverse	GCGTCATCGTATACACAGGAGC	22	
5	EeF	*eae*	Forward	TCAATGCAGTTCCGTTATCAGTT	23	482
6	EeR		Reverse	GTAAAGTCCGTTACCCCAACCTG	23	
7	BfpF	*bfp*	Forward	ATCAGTCGTCACTCACTGGT	20	300
8	BfpR		Reverse	CTGCTGTCACAGTGACAAA	19	

during 2006. Moreover, a previous study (Sikdar, 1991) reported from four different farms of North East India that 51.95 % of piglets were affected with diarrhoea where the main etiological agent was *E. coli*. The present study also relates with previous studies where *E. coli* strains were increasingly isolated from humans with diarrhoea (Nataro and Kaper, 1998; World Health Organisation Scientific Working Group, 1998). *E. coli* have many virulence factors such as attachment and effacing factors (AEF) and fimbrial adherence factor (FAF) with the help of which these bacteria attach to epithelial layer of intestine and it also produce enterotoxins (Qadri *et al.*, 2000).

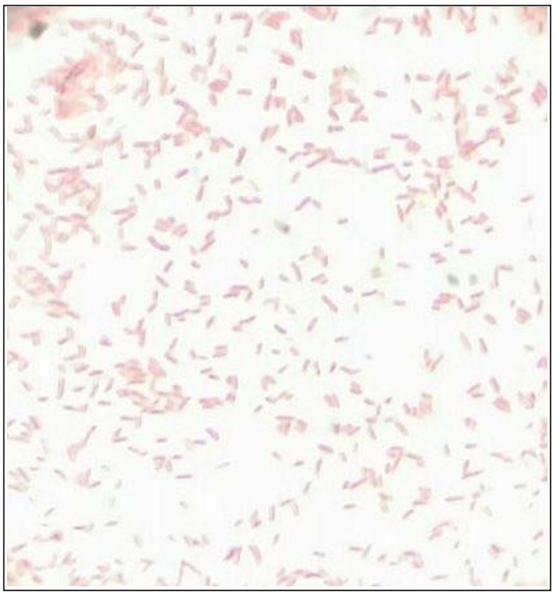

Fig. 2: Gram's stain showing Gram negative *E. coli* cells

The results of biochemical detection by Hi24 *Enterobacteriaceae* identification kit were found to be typical for *E. coli* as shown in Table 3 and Fig. 3. Moreover, in case of IMViC test, the following results were obtained as shown in Fig. 4 (a) red layer at the top of the tube after addition of Kovac's reagent giving positive result for indole test, (b) development of red colour after addition of methyl red reagent giving positive result for methyl red test, (c) lack of colour change after addition of Barritt's A and Barritt's B reagent giving negative result for Voges Proskeur test, (d) lack of growth and colour change in the tube giving negative result for citrate test. These were again typical for *Escherichia coli*.

Table 3: Biochemical detection of *E. coli* isolates

Sl. No.	Name of test	Human	Pig
1	ONPG	+	+
2	Lysine	+	+
3	Ornithine	+	+
4	Urease	-	-
5	Phenylalanine	-	-
6	Nitrate reduction	+	+
7	H_2S production	-	-
8	Citrate	-	-
9	VogesProskeur	-	-
10	Methyl Red	+	+
11	Indole	+	+
12	Malonate	+	+
13	Esculin	-	-
14	Arabinose	+	+
15	Xylose	+	+
16	Adonitol	-	-
17	Rhamnose	+	+
18	Cellobiose	-	-
19	Mellibiose	+	+
20	Saccharose	-	-
21	Raffinose	-	-
22	Trehalose	+	+
23	Glucose	+	+
24	Lactose	+	+

Fig. 3: Results shown by *E. coli* isolates on Himedia biochemical interpretation kit

Antibiotic sensitivity

The antibiotic sensitivity patterns of *E. coli* of human and pig isolates are depicted in Table 4. High resistance rates were found in case of ampicillin (74.3%) and erythromycin

(73.8%) whereas high degree of sensitivity was detected in ciprofloxacin (76.9%), chloramphenicol (77.0%) and levofloxacin (77.7%).

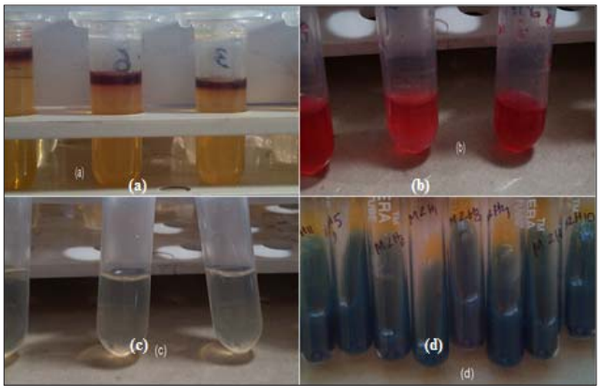

Fig. 4: Results shown by *E. coli* isolates from IMViC test, **(a)** Indole test **(b)** Methyl Red test **(c)** VogesProskeur test **(d)** Citrate test

Table 4: Antimicrobial patterns of *E. coli* isolates from humans and pigs

Sl. No.	Antimicrobial Agent	No. of isolates tested	Resistant N (%)	Intermediate N (%)	Sensitive N (%)
1	Ciprofloxacin (5mcg)	52	12 (23.0)	0	40 (76.9)
2	Chloramphenicol (30mcg)	61	10 (16.3)	4 (6.5)	47 (77.0)
3	Ofloxacin (5mcg)	74	16 (21.6)	5 (6.7)	53 (71.6)
4	Ampicillin (10mcg)	82	61 (74.3)	0	21 (25.6)
5	Erythromycin (15mcg)	42	31 (73.8)	5 (11.9)	6 (14.2)
6	Levofloxacin (5mcg)	63	3 (4.7)	11 (17.4)	49 (77.7)
7	Streptomycin (10mcg)	51	6 (11.7)	8 (15.6)	37 (72.5)

There is a worldwide increase in antimicrobial resistance in *E. coli* and it is found that its sensitivity pattern depend on geographic variation as well as differences in population and environment (Von Baum, 2000). In the present study, the overall resistance of the bacterium to antimicrobials was high which is quite close to a previous study (Orrett and Shurl, 2001). Here, high level of resistance was shown in ampicillin which relates with an earlier study (Olowe *et al.,* 2007), where it is stated that resistance of *E. coli* isolates to penicillium group of antibiotics is increasing day by day in different parts of the world. Resistance to erythromycin in the present study is similar to a previous report from Slovenia (Petkovsek *et al.,* 2009). Again, isolates were sensitive to ciprofloxacin and chloramphenicol which relates to similar studies done previously in Ethiopia (Tesfaye *et al.,* 2009) and Nigeria (Wariso, 2006). Fluoroquinolone such as levofloxacin was found to be sensitive for the *E. coli* isolates in the present study which co-relates with earlier report from different parts of the world where quinolones are found to be still active against *E. coli* infections.

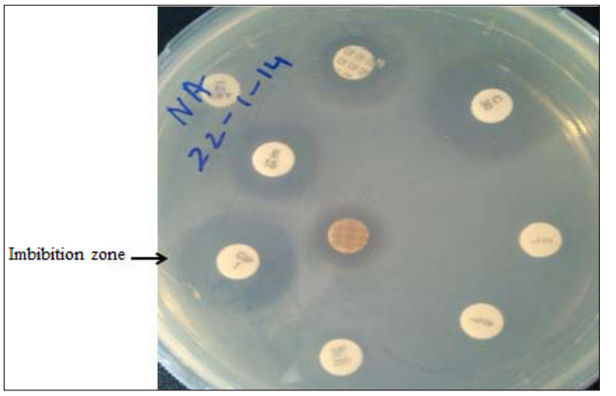

Fig. 5: NA plate showing antibiotic sensitivity test of *E. coli* isolates

Molecular detection of stx_1, stx_2, *eae* and *bfp* gene by multiplex PCR assay

In the present study, among all the isolates, only 20 human isolates harboured the *eae* gene with absence of *bfp* gene which showed that these human isolates were atypical enteropathogenic *E. coli* (EPEC). None of the pig isolates were typable due to absence of the virulence genes taken into consideration. The results are shown in Table 5 and the gel photograph of *eae* gene in human isolates is shown in Fig. 6. A previous study states that most frequently found category of diarrheagenic *E. coli* was EPEC (Vidal, 2005). It is also suggested in a previous study (Victor *et al.,* 1993) that high frequency of *eae* among strains of human origin suggests that the presence of this gene may be important

for pathogenesis of enteric disease associated with these organisms.

Moreover, it is also possible that the gene was lost during storage or a different gene is present in these *E. coli* strains which is related to *eae* but has too low homology to be detected in our PCR assay (Victor *et al.,* 1993). In the same study, it is also said that though Shiga like toxin producing *Escherichia coli* (SLT-EC) is frequently isolated from pigs, a minority of them possess *eae* gene and this may explain the lack of association of human disease and these *E. coli* strains.

Table 5: Molecular detection of stx_1, stx_2, *eae* and *bfp* genes by multiplex PCR

Species	No. of positive samples	Molecular Detection			
		No. of isolates positive for			
		Stx1	***Stx2***	***eae***	***bfp***
Human	51	0	0	20	0
Pig	42	0	0	0	0

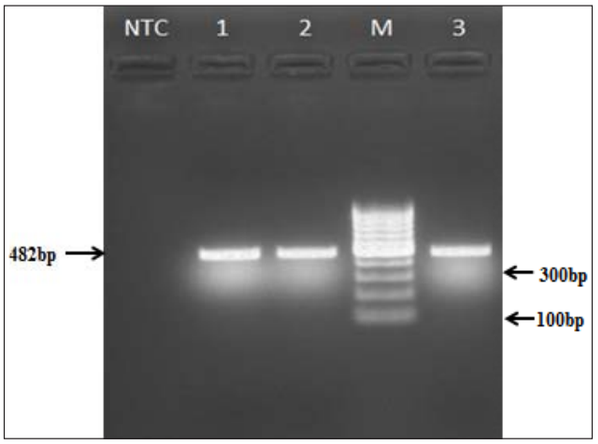

Fig. 6: Amplification of stx_1 (348bp), stx_2 (584bp), *eae* (482bp) and *bfp* (300bp) genes of *E. coli.* Lane 1, 2, 4: PCR products showing positive for eae and negative for stx_1, stx_2 and *bfp*. Lane M: DNA ladder (100bp). Lane NTC: Negative template control

CONCLUSION

Pathogenic forms of *E. coli* harbouring virulence, specific colonisation factors and pathogenicity associated genes causes a variety of diarrhoeal diseases in human and pig in North East India. In the faecal samples from human and pigs in Assam, isolated bacteria were confirmed to be *E. coli* with 80.8 percent positivity. Molecular characterisation studies targeting the virulence genes viz., stx_1, stx_2, *eae* for shiga toxin producing *E. coli* (STEC) also confirmed presence of enteropathogenic *E. coli* (EPEC) among the positive isolates aiming the *eae* and *bfp* genes. Among the human isolates, 39.21 percent were EPEC and none contained the *bfp* gene, signifying as atypical EPEC. However, none of the pig isolates were typable. Results of this baseline study on pathogenic form of *E. coli* may help in further serogrouping of the organism for diagnostics and pathogenesis implications.

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