Detection of Escherichia coli Pathotypes in Children with Diarrhea


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ABSTRACT

Escherichia coli is one of the most important causes of diarrheal illness in infants and children. Therefore a study was undertaken to know the occurrence of E. coli pathotypes in diarrheal cases of children. Stool samples (total 100) were immediately processed for isolation and identification of E. coli. Biochemically confirmed isolates were screened for different pathotypes viz., shigatoxic E. coli (STEC), enterotoxigenic E. coli (ETEC), enteropathogenic E. coli (EPEC), and enteroinvasive E. coli (EIEC) by an PCR targeting virulence genes viz., stx1 and stx2, lth, eaeA and bfpA, respectively. Of the 32 confirmed E. coli, 24 were diarrheagenic strains. The frequency of distribution was recorded as STEC (15/46.87%), ETEC (12/37.50%) and EPEC (6/18.75%). None of the isolate was positive for hly gene specific to EIEC. Six (18.75%) E. coli strains harboured multiple virulent genes of different pathotypes. E. coli isolates were susceptible to ciprofloxacin (90.62%), ceftriaxone (90.62%), cephotoxime (87.5%), enerofoxacin (84.37%) and norfoxacin (81.25%), while few strains expressed resistance to ampicillin (43.75%), cloxacin (40.75%), tetracycline (34.37%) and oxytetracycline (21.87%). Further surveillance studies are required to investigate the ecological, socioeconomic and epidemiological basis of diarrheagenic E. coli with special reference to STEC strains and their association with human diarrheal illness.

Keywords: Antimicrobial susceptibility, children, diarrhea, E. coli pathotypes

Introduction

Diarrheal diseases account for one in nine child deaths worldwide, making it the second leading cause of death among children under the age of five (Liu et al., 2012). Many cases are not diagnosed, either because they are mild self limiting, patients do not seek medical attention or because medical and laboratory sources are not available (Quadri et al., 2005). Diarrhea is a multi-etiological illness and more than 20 viral, bacterial and parasitic agents are associated with acute diarrhea. Rotavirus and diarrheagenic Escherichia coli (DEC) are most commonly responsible for acute diarrhea in children (Nguyen et al., 2005). Although, E. coli are the normal inhabitants of the intestinal tract and also indicators of fecal contamination, few strains are pathogenic. Based on their virulence, DEC strains are grouped into different pathotypes viz., enterotoxigenic E. coli (ETEC), enteropathogenic E. coli (EPEC), enteroinvasive E. coli (EIEC), enterohemorrhagic E. coli (EHEC), enteroaggregative E. coli (EAEC) and diffusely adhering E. coli (DAEC). All of them have potential to cause mild to severe illness including diarrhea (Nataro and Kaper, 1998). During the past decades, STEC has evolved from clinical novelty to a global public health concern, which can cause severe bloody diarrhea and even life threatening sequelae such as haemolytic uremic syndrome. The ability of STEC to cause serious disease in human is related to the production of one or more shiga-like toxins (Stx1, Stx2, or their variants), which inhibits protein synthesis of host cell leading to cell death (Khan et al., 2002). E. coli are widespread in nature with animal reservoirs for some of the pathotypes notably, STEC. In India STEC strains are still not considered as potential cause of human diarrhea as that of enteropathogenic and enterotoxigenic strains (Khan et al., 2002; Dhanashree and Mallya, 2008). There are several modes through which E. coli infection may be acquired by children. Present investigation was conducted with aim to assess the prevalence of DEC in stool samples of children suffering from diarrhea.

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Materials and Methods

Sampling

About 10 g of stool sample from children suffering from diarrheal illness were provided by pediatricians for investigation. A total of 100 samples were procured and processed for isolation and identification of DEC strains in the laboratory of Department of Veterinary Public Health, KNP College of Veterinary Science, Shirwal. All the samples were transported to laboratory under low temperature and immediately processed.

Isolation and identification

Stool samples were diluted 1:10 in enterobacteria enrichment broth (EEB) and incubated. A loopful from EEB was streaked on eosin methylene blue (EMB) agar and incubated. Colonies showing typical metallic sheen on EMB agar were further streak on MUG-sorbitol agar and again incubated. All incubations were carried at 37°C for 24 h. Pale coloured colonies from MUG-sorbitol were subjected for biochemical characterization viz., catalase, oxidase, indole, methyl red, Voges Proskauer tests (Agarwal et al., 2003).

Antibiogram

Antibiotic susceptibility profiles of E. coli isolates were studied against 16 different antibiotics according to the method described by Bauer et al. (1966). Briefly, 18 h culture of test organism was evenly spread on Muller Hinton agar (MHA) plates and allowed to dry. Different antibiotic discs were placed on MHA and incubated at 37°C for 18-24 h. Results in terms of sensitive, moderate sensitive or resistant were interpreted as per the manufacturer’s guidelines (HiMedia, 2009).

Detection of DEC strains

Pathotype specific virulent genes were targeted using previously described PCR protocols (Nguyen et al., 2005; Ratchtrachenchai et al., 2004; Jakee et al., 2009) with suitable modifications. The genomic DNA of E. coli was extracted by heat lysis and subjected to PCR with specific primers for the detection of virulence genes viz., lth, Stx1, Stx2, eaeA, bfpA and hly (Table 1). The PCR was performed with a final reaction volume of 25 µL using containing 12.5 µL 2x PCR master mix (Thermo Scientific), 0.5 µL each forward and reverse primers, 3 µL DNA template and 8.5 µL nuclease free water. All above components were mixed by gentle shaking and tubes were subjected for PCR in thermal cycler (Eppendorf, Germany). Five microliter amplified PCR product was further separated by electrophoresis in 2% agarose gel stained by ethidium bromide. After electrophoresis gel was visualized under a UV transilluminator (G-BOX F3 Syngene). GeneRuler™ 100 bp DNA Ladder (MBI Fermentas, USA) was used as a marker.

Results and Discussion

Thirty two E. coli were isolated using MUG sorbitol agar. Pathogenic strains do not ferment sorbitol and exhibits pale colour colonies; however, sorbitol fermenting E. coli exhibits pink coloured colonies on MUG sorbitol agar. Presence of E. coli in stool either from clinical or non clinical subject is obvious, since they are normal inhabitant of gut microflora. However, presence of DEC E. coli in stool is of public health significance.

Fig. 1. Detection of E. coli pathotypes by PCR.
Lane I: 100 bp DNA ladder, Lane II and III: stx2 gene (228 bp), Lane IV and V: stx1 gene (130 bp), Lane VI and VII: eaeA gene (488 bp), Lane VIII and IX: lth gene (132 bp), Lane X and XI: bfpA gene (234 bp)
Table 1. PCR primers and PCR conditions used for detection of DEC pathotypes

<table>
<thead>
<tr>
<th>DEC type</th>
<th>Genes targeted</th>
<th>Oligonucleotide sequence</th>
<th>PCR conditions</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHEC</td>
<td>stx1</td>
<td>(F) GAA GAG TCC GTG GGA TTA CG (R) AGC GAT GCA GCT ATT AAT AA</td>
<td>D: 94°C/20 sec A: 55°C/20 sec E: 72°C/10 sec</td>
<td>130</td>
<td>Nguyen et al. (2005)</td>
</tr>
<tr>
<td>EHEC</td>
<td>stx2</td>
<td>(F) ACC GTT TTT CAG ATT TTG A CAC ATA (R) TAC ACA GGA GCA GCT TCA GAC AGT</td>
<td></td>
<td>228</td>
<td></td>
</tr>
<tr>
<td>ETEC</td>
<td>lth</td>
<td>(F) AGC AGG TTT CCC ACC GGA TCA CCA (R) CGT GCT CAG ATT CTG GGT CTC</td>
<td>D: 94°C/1 min A: 48°C/1.5 min E: 72°C/2 min</td>
<td>132</td>
<td>Ratchrachenchai et al. (2004)</td>
</tr>
<tr>
<td>EPEC</td>
<td>eaeA</td>
<td>(F) GCT TAG TGC TGG TTT AGG AT (R) TCG CCG TTC AGA GAT CGC</td>
<td></td>
<td>488</td>
<td></td>
</tr>
<tr>
<td>EPEC</td>
<td>bfpA</td>
<td>(F) GAA GTA ATG AGC GCA ACG TC (R) ACA TGC CGC TTT ATC CAA CC</td>
<td></td>
<td>234</td>
<td></td>
</tr>
<tr>
<td>EIEC</td>
<td>hly</td>
<td>(F) AGC ATG TGG TTT ATT CTG (R) CTT CAC GTG ACC ATA CAT AT</td>
<td>D: 94°C/1 min A: 46°C/1.5 min E: 72°C/2 min</td>
<td>165</td>
<td>Jakee et al. (2009)</td>
</tr>
</tbody>
</table>

D: Denaturation, A: Annealing, E: Extension

Table 2. Antibiogram of *E. coli* isolates (n = 32)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of antibiotic with concentration</th>
<th>Sensitive (%)</th>
<th>Moderate Sensitive (%)</th>
<th>Resistant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ampicillin (10mcg/disc)</td>
<td>09.37</td>
<td>46.87</td>
<td>43.75</td>
</tr>
<tr>
<td>2</td>
<td>Amikacin (10mcg/disc)</td>
<td>75.00</td>
<td>25.00</td>
<td>00.00</td>
</tr>
<tr>
<td>3</td>
<td>Chloramphenicol (10mcg/disc)</td>
<td>65.62</td>
<td>25.00</td>
<td>09.37</td>
</tr>
<tr>
<td>4</td>
<td>Cephotaxime (10mcg/disc)</td>
<td>87.50</td>
<td>06.25</td>
<td>06.25</td>
</tr>
<tr>
<td>5</td>
<td>Ciprofloxacin (10mcg/disc)</td>
<td>90.62</td>
<td>09.37</td>
<td>00.00</td>
</tr>
<tr>
<td>6</td>
<td>Ceftriaxone (10mcg/disc)</td>
<td>90.62</td>
<td>09.37</td>
<td>00.00</td>
</tr>
<tr>
<td>7</td>
<td>Cephalexin (30mcg/disc)</td>
<td>53.12</td>
<td>43.75</td>
<td>03.12</td>
</tr>
<tr>
<td>8</td>
<td>Chlorotetracycline (30mcg/disc)</td>
<td>12.50</td>
<td>62.50</td>
<td>25.00</td>
</tr>
<tr>
<td>9</td>
<td>Cloxacillin (10mcg/disc)</td>
<td>28.12</td>
<td>18.75</td>
<td>53.12</td>
</tr>
<tr>
<td>10</td>
<td>Erythromycin (10mcg/disc)</td>
<td>18.25</td>
<td>71.87</td>
<td>9.37</td>
</tr>
<tr>
<td>11</td>
<td>Enerofloxacin (10mcg/disc)</td>
<td>84.37</td>
<td>15.62</td>
<td>00.00</td>
</tr>
<tr>
<td>12</td>
<td>Gentamicin (10mcg/disc)</td>
<td>50.00</td>
<td>40.62</td>
<td>9.37</td>
</tr>
<tr>
<td>13</td>
<td>Norfloxacin (10mcg/disc)</td>
<td>81.25</td>
<td>18.75</td>
<td>00.00</td>
</tr>
<tr>
<td>14</td>
<td>Oxytetracycline (30mcg/disc)</td>
<td>09.37</td>
<td>68.75</td>
<td>21.87</td>
</tr>
<tr>
<td>15</td>
<td>Streptomycin (10mcg/disc)</td>
<td>37.50</td>
<td>40.62</td>
<td>21.87</td>
</tr>
<tr>
<td>16</td>
<td>Tetracycline (10mcg/disc)</td>
<td>31.25</td>
<td>34.37</td>
<td>34.37</td>
</tr>
</tbody>
</table>

Several literatures worldwide highlighted the role of pathogenic *E. coli* in human diarrheal illness (Nishikawa et al., 2002; Vidal et al., 2004; Wagner et al., 2004; Seto et al., 2007; Dhanashree and Mallya, 2008).

The purpose of present investigation was to assess the prevalence of pathogenic *E. coli* in childhood diarrhoeal cases. Identification of diarrheagenic *E. coli* strains requires the differentiation of these organisms from non pathogenic members of the genus. Specific serotyping is not always correlated with pathogenicity. PCR is commonly used method that gives rapid and reliable results and shows high sensitivity and high specificity (Garcia et al., 2011). Therefore, all *E. coli* isolates under the study were screened by PCR targeting various virulent genes viz., *stx1*, *stx2* for STEC, *lth* for ETEC, *eaeA*, *bfpA* for EPEC, *hly* for EIEC. Of the 32 *E. coli*, 24 (75%) were confirmed as DEC strains. Out of 24 DEC, virulent pathotypes detected were EPEC (25%), ETEC (50%), STEC (62.5%) and EIEC (0%). Surprisingly, prevalence of STEC was recorded more than ETEC and EPEC. Begum et al. (2013) reported the prevalence of STEC in cattle was 16.21% (12 out of 74) in which nine isolates were positive for both *stx1* and *stx2* genes, two were positive for *stx1* gene and 1 was positive for *stx2* gene, whereas none of the isolates were found positive for *eaeA* and *hly* gene. Worldwide literatures have highlighted the dominance of EPEC and ETEC in human diarrhea, and STEC strains.
are rarely isolated (Phantoumath et al., 2003; Nguyen et al., 2005; Bueris et al., 2007). Chattopadhyay et al. (2003) made an attempt to isolated and characterized STEC from diarrheic children, cattle, animal handlers, beef and pork. STEC could be isolated by them from diarrheic cattle (22.07%) and diarrheic children (1.33%). In India, although STEC has not been identified as a major etiological cause of diarrhoea it has been isolated from diverse sources suggesting that this enteropathogen may pose a public health problem (Khan et al., 2002; Dhanashree and Mallya, 2008). Relatively lower prevalence of EPEC (6%), ETEC (7.8%), and EAEC (3.7%) among hospitalized patient from Orissa was also recorded by Samal et al. (2008). Nishikawa et al. (2002) observed the distribution of 67 DEC stains as EPEC (26%), EHEC (15%), ETEC (6%) and eaeA (20%) isolated from sporadic cases of diarrheal illness in Osaka city Japan. Except for EAEC, their findings revealed rising tendency in summer. These findings strongly supports results of present investigation wherein high prevalence of STEC and ETEC recorded and all stool samples were collected during winter to summer months.

E. coli strains exhibited resistance to ampicillin (43.75%), cloxacillin (53.12%), and tetracycline (34.37%) and less frequently to oxytetracycline (21.87%) and streptomycin (18.87%). They were moderately sensitive to erythromycin (71.87%), oxytetracyclin (68.75%), chlorotetracycline (62.50%) and ampicillin (46.87%). Isolates were highly sensitive to ciprofloxacin (90.62%), ceftriaxone (90.62%), cephoxatime (87.5%), enerofoxacin (84.37%) and norfloxacin (81.25%) (Table 2). Antibiogram of DEC revealed maximum resistance to tetracycline (45-83%), cloxacillin (41.66%) and ampicillin (37.5%). These observations are in agreement with some of the previous findings (Khan et al., 2002; Schroeder et al., 2002; Chigor et al., 2010; Garcia et al., 2011). Schroeder et al. (2002) suggested that selection pressure imposed by the use of tetracycline derivatives, sulphad drugs,cephalosporins and penicillin therapeutically in human and veterinary medicine or as prophylaxis in the animal production environment is a key driving force in the selection of antimicrobial resistance in STEC and non-STECC 0157 strains. They recorded tetracycline (26%) and ampicillin (13%) resistance in E. coli isolates from human diarrheal cases. Tetracycline is infrequently used to treat human enteric infection yet a substantial number of human E. coli isolates were tetracycline resistant. Although, it is rarely used in human, antibiotic resistance gene pool generated in animal production systems may be mobile and persistent in the environment with the potential to enter the food chain as suggested by Aminov et al. (2002). Bettelheim et al. (2003) also observed tetracycline as the most common antibiotic against which VTEC from humans showed resistance. Prevalence of antibiotics resistance among the strains isolated from babies less than one year of age who had not much exposure to antibiotics is also observed in the study. Such resistant E. coli may have originated from mothers faecal flora or hospital environment as suggested by Bettelheim et al. (2003). Present findings are also in accordance with Khan et al. (2002) who observed resistant STEC strains most commonly to ampicillin (25%), tetracycline (23.8%) and streptomycin (14.3%). Multidrug resistance was seen and there was no common resistance pattern among strains. Alhaj et al. (2007) revealed maximum resistance to tetracycline, kanamycin, chloramphenicol and gentamicin in E. coli isolated from human and environmental sources in Malaysia. Chigor et al. (2010) also noted resistance in E. coli isolates from stool samples to ampicillin (80.7%) and tetracycline (61.4%). Shalaya et al. (2013) conducted community based survey of E. coli antimicrobial resistance in children from Ujjain and 33% isolates were multi drug resistant. The proportion of isolates resistant was: nalidixic acid (45%), tetracycline (37%), ampicillin (37%) and amoxicillin/clavulanic acid (29%). Variation in isolation rate of E. coli pathotypes and their antimicrobial resistance pattern is obvious and attributed to the source of isolation, locality, socioeconomic status and environmental hygiene. Findings of present study clearly indicates the need of detailed microbial surveillance of DEC strains from large population comprising of diarrheal and healthy subjects to establish the epidemiology of STEC and other DEC strains and their association of children diarrhea.

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References


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