Original Article

Typical and atypical enteropathogenic Escherichia coli in children with acute diarrhoea: Changing trend in East Delhi

K. Sneha a, Taru Singh a, Sajad Ahmad Dar a,b, Shaful Haque b, Vishnampetai G. Ramachandran a, Rumpa Saha a, Dheeraj Shah c, Shukla Das a,*

a Department of Microbiology, University College of Medical Sciences (University of Delhi), Guru Teg Bahadur Hospital, Delhi, India
b Research and Scientific Studies Unit, College of Nursing & Allied Health Sciences, Jazan University, Jazan, Saudi Arabia
c Department of Paediatrics, University College of Medical Sciences (University of Delhi), Guru Teg Bahadur Hospital, Delhi, India

ARTICLE INFO

Article history:
Received 21 November 2018
Accepted 16 March 2020
Available online 21 April 2020

Keywords:
Enteropathogenic Escherichia coli (EPEC)
E. coli attaching and effacing gene (eae)
Bundle forming pilus (bfp)
Diarrhoea
Children

ABSTRACT

Background: Worldwide around 2 million deaths occur every year due to diarrhoeal illnesses among children less than 5 years of age. Among diarrhoeagenic Escherichia coli, Enteropathogenic E. coli (EPEC) is highly prevalent in both community and hospital settings and is one of the main causes of persistent diarrhea in children in developing countries. EPEC remains underdiagnosed in India due to lack of conventional tools for identification.

Methods: We in this study investigated the prevalence and regional variation of EPEC in paediatric population suffering from diarrhoea in East Delhi, India. Two hundred stool samples were collected from children, aged between 0.5 and 5 years, with acute diarrhoea. E. coli were identified by conventional tests and PCR.

Results: We observed 7% atypical EPEC (aEPEC) and 2.5% typical EPEC (tEPEC), with an overall 9.5% EPEC prevalence amongst total samples. E. coli phylogenetic group A was the predominant. The most common age group affected was 6–23 months with common symptoms being vomiting, watery diarrhoea and severe dehydration. High drug resistance pattern was observed in EPEC isolates.

Conclusion: The study depicts a changing trend of aEPEC over tEPEC in children less than 5 years with diarrhoea, an emerging drug resistant enteropathogen and a public health concern demanding monitoring and surveillance.

* Corresponding author. Department of Microbiology, University College of Medical Sciences (University of Delhi), Guru Teg Bahadur Hospital, 2, Tahirpur Rd, GTB Enclave, Dilshad Garden, New Delhi, Delhi 110095, India.
E-mail address: shukladas_123@yahoo.com (S. Das).
Peer review under responsibility of Chang Gung University.
https://doi.org/10.1016/j.bj.2020.03.011
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Diarrhoeal illnesses are a major public health problem particularly among children less than 5 years of age with deaths of over 2 million occurring every year in this age group [1]. Diarrhoeagenic *Escherichia coli* (DEC) is one of the pathogens and important causes of infantile diarrhoea affecting developing nations [2].

Among the DEC pathotypes, Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC) and Enteraggregative *E. coli* (EAEC) are the most important pathogens and an important cause of infantile diarrhoea infecting children worldwide [3–5].

EPEC is classified into typical and atypical strains depending upon *eae* gene located in the ‘locus of enterocyte effacement’ (LEE) and *bfpA* gene on a plasmid called ‘EPEC adherence factor’ (EAF). Typical EPEC (tEPEC) strains are *eae*+ and *bfpA*+ whereas atypical EPEC (aEPEC) strains possess *eae* but lack *bfpA* i.e. *eae*+ *bfpA*− [6].

The identification of DEC is difficult as these cannot be adequately diagnosed by culture and biochemicals alone. EPEC may be diagnosed by serological tests but these tests are expensive and laborious; also all the strains may not be typable or belong to the same or the existing pools of serovars. These pathogens can be easily diagnosed by molecular diagnostic techniques such as PCR [7,8]. Classification of DEC as well as its molecular identification is established on the basis of presence or absence of specific virulence genes [9].

Infantile diarrhoea due to EPEC in India remains under-diagnosed due to lack of conventional tool for identification. Although the events are self-limiting, identification helps the clinicians to decide upon the course of management. PCR is now a commonly used method for rapid and reliable identification with high sensitivity and specificity, and for detecting various genes coding for virulence in different categories of DEC [10]. PCR-based detection provides a renewed opportunity to look for the epidemiology of EPEC strains in developing countries where EPEC is a major cause of infantile diarrhoea.

This study was undertaken because EPEC is highly prevalent in both community and hospital settings in developing countries including India, and it is one of the main causes of persistent diarrhea in children [11,12]. The aim of the study was to utilize PCR-based detection for the commonest phylogroups of EPEC acting as the etiological agents of diarrhoea in children less than five years old, and also to analyse the antibiotic resistance pattern of these isolates, in East Delhi region.

### Materials and methods

#### Study samples

This cross-sectional study was conducted in the Department of Microbiology and Paediatrics, at an 1800 bedded tertiary care hospital in East Delhi, India. Written informed consent was taken from the parents/guardians of the study population before collection of stool samples. The study was reviewed and approved by the Institutional Ethical Committee-Human Research (IEC-HR). The study spread over a period of 12 months.

Two hundred children aged between 6 months and 5 years presenting to the paediatric outpatient/emergency departments with acute diarrhoea irrespective of the state of dehydration were enrolled for stool sample collection during June, 2014 to June, 2015. An episode of acute diarrhoea was defined as passage of three or more loose stools, liquid or watery over a period of 24 h for a maximum duration of 7 days. Children who had received antibiotics or any anti-diarrheal drug in the preceding 96 h or were receiving antibiotics for the current episode of diarrhoea or for any unrelated disease were excluded. A detailed history regarding the demography and the symptoms pertaining to diarrhoea was taken along with the general physical examination.

#### Table 1 Primers used to detect virulence genes specific for EPEC.

| Target gene | Primers | Amplicon size (bp) | Reference(s) |
|-------------|---------|--------------------|--------------|
| Eae         | Forward | 5’TCAATGCGATTCGGTTATCAGTT3’ | 482          | [14]  |
|             | Reverse | 5’GTAAAGTCGGTTACCCCAACCTG3’ |              |      |
| bfpA        | Forward | 5’AAATGGTCTCCTGGCAGCCTG3’ | 326          | [15]  |
|             | Reverse | 5’GCCGCTTTATCACAACCTGTA3’ |              |      |
| GAPDH       | Forward | 5’ACTTACGCACAGAGCAGACG3’ | 170          | [16]  |
|             | Reverse | 5’AGTTTCAGGAAGTGTCTG3’ |              |      |

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**At a glance of commentary**

### Scientific background on the subject

High prevalence of EPEC in community and hospital settings in developing countries like India, and its underdiagnoses in infantile diarrhoea, is mainly responsible for persistent diarrhea in children. We utilized PCR-based detection for commonest EPEC phylogroups acting as etiological agents of diarrhoea in children less than five, and analyzed their antibiotic.

### What this study adds to the field

The study demonstrates aEPEC as a more prevalent pathogen than tEPEC in children with acute diarrhea in East Delhi region of India. The *Escherichia coli* phylogenetic group A was found predominant suggesting adaptive advantages and acquired pathogenicity in aEPEC, similar to that observed in the industrialized countries.

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Conventional culture

Stool samples were collected and inoculated onto MacConkey agar. After overnight incubation at 37 °C, lactose fermenting colonies from MacConkey agar were identified by conventional biochemical tests for identification of E. coli. Conventional biochemical tests for identifying E. coli were gram staining (gram-negative, rod-shaped bacterium), catalase test (+ve), oxidase test (-ve), glucose fermentation with production of gas, fermentation of other sugars (lactose, sucrose, maltose and mannitol), nitrate reduction (+ve; reduces nitrate into nitrite), urease (-ve), methyl red (+ve) and VogesProskauer (-ve), OF glucose test (glucose fermenter), decarboxylase test [lysine (+ve), arginine (-ve) and ornithine (+ve/-ve)], indol test (+ve), Simon’s citrate (-ve) and hydrogen sulfide (-ve) [13].

**DNA extraction and PCR**

Four to five lactose fermenting colonies phenotypically confirmed as E. coli were picked for DNA extraction (HiYield™ Genomic DNA Mini Kit from BioAmerica Inc., Miami, FL, USA) and this DNA was later used as a template for multiplex PCR to detect virulence genes using primers specific for EPEC (Table 1) [14–16]. The amplification protocol was as follows: initial denaturation at 95 °C for 5 min, cyclic denaturation at 95 °C for 40 secs, annealing at 53 °C for 35 secs, extension at 72 °C for 40 secs and final extension at 72 °C for 7 min. The E. coli isolates were then characterized into typical and atypical variants of EPEC. The EPEC isolates which were positive for both eae and bfpA were grouped in typical EPEC and those with eae+ but bfpA− were grouped in atypical EPEC. The E. coli reference strain from National Institute of Cholera and Enteric diseases, Kolkata, India was used as positive control (E. coli ATCC 43887 EPEC eae+/bfpA+/eaeA+) and non-pathogenic E. coli ATCC 25922 was used as negative control. The primers for amplification of GAPDH (size 170bp) gene were used for amplification internal quality control. A molecular marker (100bp DNA ladder) was used to determine the size of the amplicons.

A quadruplex PCR was performed to distribute the isolates among seven phylogroups as described by Clermont et al., 2013 [17].

**Antibiotic sensitivity**

All the samples were subjected to antibiotic susceptibility testing for ampicillin (10 μg), nalidixic acid (30 μg), norfloxacin (10 μg), gentamicin (10 μg), amikacin (30 μg), cefotaxime (30 μg), imipenem (10 μg), aztreonam (30 μg), and piperacillin/tazobactam (100/10 μg). The E. coli ATCC 25922 was used as a quality control strain for antimicrobial susceptibility testing by the disc diffusion method as per Clinical and Laboratory Standards Institute (CLSI) guidelines [18].

**Statistical analysis**

Statistical analysis was done using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Comparisons of proportions were conducted using chi-square test. Data was presented as either percentage of the total or mean ± SD, and median along with the minimum and maximum values, wherever appropriate.

**Results**

EPEC was found to be the joint most common pathogen along with *Vibrio cholerae* (19 each; 9.5%) associated with diarrhoea among 200 acute diarrhoeal children. The characterization of E. coli into one of the pathotypes of DEC i.e. EPEC, and subsequent grouping into tEPEC and aEPEC, based on the presence of eae and bfpA genes, revealed 7 percent prevalence of aEPEC (eae+, bfpA−) and 2.5 percent prevalence of tEPEC (eae+, bfpA+) with an overall 9.5 percent prevalence of EPEC among the total acute diarrhoeal cases (Fig. 1). This identifies aEPEC as the most common cause for diarrhoea in these children. The most common age group affected among the children was 6–23 months. Atypical EPEC was observed to be common in 6–12 months compared to tEPEC which was common among 13–23 months aged children. Vomiting, watery diarrhoea and severe dehydration were observed to be the common symptoms with EPEC infection (Table 2).

Severe dehydration was observed commonly among the EPEC-positive children as well as in both tEPEC (60%) and aEPEC (78.57%) diarrhoea. Among all EPEC-positive cases,
vomiting was the most common symptom followed by fever. Watery diarrhoea was predominant among EPEC-positive patients and was present in all the cases of tEPEC and aEPEC except one patient with tEPEC which was associated with blood and mucus. Less severe symptoms and complications were observed in diarrhoeic children infected with other enteric pathogens. There was no difference in the age group of children suffering from diarrhoea due to EPEC or cholera though the degree of dehydration was conspicuous in cholera, up to 90% with severe dehydration as compared to 78.57% in EPEC group.

| Variables                        | Children with EPEC-positive diarrhoea (n = 19) | p-value |
|----------------------------------|------------------------------------------------|---------|
| 1. Age in months, Mean (SD)      | aEPEC, n = 14; 20.21 (13.16) | tEPEC, n = 5; 30.40 (19.76) | – |
| 2. Age group                     | 2–5 years (4 [28.57%]) | 2–5 years (4 [40%]) | 1.000 |
| 3. Sex                           | Males (7 [50%]) | Males (3 [60%]) | 1.000 |
| 4. Source of drinking water      | Piped water (12 [85.71%]) | Others (14.28%) | 1.000 |
| 5. Breast feeding                | On Breast feeding (5 [35.71%]) | Not on breast feeding (4 [80%]) | 1.000 |
| 6. Dehydration status            | Severe dehydration (11 [78.57%]) | Some dehydration (3 [40%]) | 0.570 |
| 7. Vomiting                      | Present (11 [78.5%]) | Absent (3 [21.5%]) | 0.530 |
| 8. Fever                         | Present (3 [21.5%]) | Absent (11 [78.57%]) | 1.000 |
| 9. Duration of diarrhoea         | Less than 2 days (6 [42.8%]) | More than 2 days (8 [57.14%]) | 1.000 |
| 10. Stool consistency            | Watery (14 [100%]) | Watery, blood, mucoid (0 [0%]) | 0.263 |
| 11. Nutritional status           | WHZ score, Mean (SD); Median (Max., Min.) | WAZ score, Mean (SD); Median (Max., Min.) | HAZ score, Mean (SD); Median (Max., Min.) | MAC <11.5 cm |
|                                 | –2.183 (1.039); –2.03 (–0.64, –5.11) | –2.477 (1.132); –2.31 (–1.14, –4.93) | –1.94 (0.49, –3.85) | 1 (7.14%) |
|                                 | –1.516 (1.066); –1.390 (0.05, –2.68) | –1.912 (0.775); –2.18 (–0.96, –2.72) | –1.27 (–0.26, –3.35) | 0 (0%) |

There was no statistically significant difference observed between the children having aEPEC and tEPEC isolates for all the parameters at 5% level of significance.

Abbreviations: WHZ score: Weight for height Z score; WAZ score: Weight for age Z score; HAZ score: Height for age Z score; MAC: Mid arm circumference.

Table 2 Comparison of the clinical and demographic features of cases of diarrhoea due to typical and atypical EPEC.

Discussion

Diarrhoegenic E. coli are amongst major bacterial causes of diarrhoea in children worldwide [21,22]. The typical variant of EPEC is reported to be a leading cause of infantile diarrhoea in developing countries, whereas the atypical variant is an important cause of diarrhoea in industrialized countries [23,24]. Although other enteropathogens were also isolated from our study samples, we are limiting our discussion to (21%) and imipenem and aztreonam (15% each) as shown in Fig. 3. The resistance to cefotaxime and imipenem were confirmed by specific confirmatory tests like double disc synergy test (DDST) for extended spectrum β-lactamases (ESBL) production [19], and Modified Hodge test for carbapenemase production [20] and they were found in complete concordance with the screening tests (data not shown).
EPEC only. We in this cross sectional study determined the prevalence of typical and atypical variants of EPEC among the children less than 5 years of age from the East Delhi region of Northern India. The results demonstrated an overall prevalence of 9.5% of EPEC in children with acute diarrhoea. The prevalence of aEPEC was 7% and tEPEC was 2.5% in children with acute watery diarrhoea demonstrating that aEPEC was more common than tEPEC. Similar prevalence rates have been reported from various studies in India: a range from 10.4% to 8% in Mangalore [9,25], 7.97% in Kashmir [26] with aEPEC being more common than tEPEC. Globally many studies with similar prevalence rates of EPEC such as 6.6% in Vietnam [27], and 9.5% in Brazil [10] have been observed. Lower prevalence rates have also been reported in studies from Thailand (3.2%) and Tanzania (4.6%) [28,29]. Some studies have shown a drastic decline in the frequency of tEPEC, with a rise in the aEPEC strains [10,30]. For many years, infections with aEPEC were thought to predominate in developed nations while being relatively rare in the developing world. However, recent data indicates that infections with aEPEC are more common than tEPEC in both developing and developed countries. Such variations in geographic distribution are identified in industrialised countries, where a decline in the occurrence of tEPEC has occurred due to reasons not very well defined.

The role of EPEC has often been ignored because of poor detection methods in routine laboratories in developing countries like India. The current study demonstrates a higher prevalence rate of 9.5% EPEC in comparison to a previous study by Ghosh et al., 2010 [31] in Northern India reporting 4.05% of EPEC among children with diarrhoea, the majority being tEPEC (75%). Atypical EPEC (73.68%) identified as a dominant EPEC pathogen contrasting other studies. Dutta et al. reported a prevalence of 1.8% for EPEC with almost the same prevalence of typical and atypical isolates among the children with diarrhoea, which again demonstrates a changing trend and the appearance of aEPEC emerging as the dominant pathogen compared to tEPEC which is a more virulent pathogen [32]. We observed that overall, EPEC patients presented with vomiting, watery diarrhoea and severe dehydration as common symptoms. This observation is consistent with that of previous studies, which recorded similar symptoms in EPEC infection [32]. Comparison of demographic profile of children suffering from diarrhoea due to EPEC or other bacterial agents, especially cholera, has been reported. The affected age of occurrence is mostly <24 months in EPEC diarrhoea whereas cholera affects on an average 36–96 months age group, though the clinical presentations may not vary [33].

The variations in the prevalence of EPEC may be influenced by multiple factors which determine the virulence potential of tEPEC and aEPEC. Firstly, the typical and atypical isolates have been characterized as highly heterogeneous groups. The heterogeneity observed may be virtual than real and stems from a lack of specified virulence factors or genomic features which are used as signature characteristics when mobile genetic elements such as bacteriophages or plasmids encode these factors. Atypical EPEC have been defined on the basis of presence or absence of virulence genes most of which are present on the mobile genetic elements [34]. Secondly there
could be horizontal gene transfer of these transmissible plasmids, pathogenicity islands, transposons or bacteriophages leading to emergence of different combinations of virulence gene sequences in aEPEC [35].

It has been also seen that aEPEC have an innate property to persist longer in the gut epithelial cells and disrupt the normal cellular process (may decrease apoptosis of intestinal epithelial cells) [36]. Certain findings also suggested that aEPEC may decrease the intestinal apoptosis possibly due to the lack of bfpA which may favour the prolonged intestinal colonisation in comparison to other intestinal pathogens [22]. However reports of aEPEC being significantly associated with endemic diarrhoea and outbreaks are also documented [21]. The pathogenic role of the invasiveness of EPEC is unknown and this characteristic may contribute to the prolonged sojourn of these strains in the intestine leading to diarrhoea under favourable host conditions, as reported by Afset et al., 2004 [6], Nguyun et al., 2006 [37] and Nair et al., 2010 [38]. Serine protease autotransporters of the Enterobacteriaceae (SPATE) and pic gene in aEPEC can confer adaptive advantages and additional pathogenic mechanisms to the attaching and effacing (A/E) lesion. This may contribute to invasion and immune evasion of aEPEC in systemic infections [39]. We have also observed this changing and emerging trend during diarrhoeal disease surveillance of our paediatric populations from east Delhi. The possibility that the atypical strains possess additional virulence factors or involve certain host factors which in association with these strains can cause disease, remains to be identified [40].

The E. coli populations are structured in seven major phylogenetic groups A, B1, B2, C, D, E and F [17]. In this study, there was predominance of phylogenetic group A (nine) followed by phylogenetic group B1 (three), group D (three), group F (two), group B2 (one) and group C (one). Phylogenetic studies are important to improve the understanding of E. coli populations, the relationship of strains, their hosts and disease, and established link between phylogenetic group and virulence. The phylogenetic classification observed suggests that the commensal strains might have acquired the virulence genes and become pathogenic.

A study from China has indicated that humans and animals, including food-producing animals and pet animals, act as reservoirs of aEPEC while the major reservoirs of tEPEC are humans [41]. Under these circumstances, adaptive advantages and additional pathogenic mechanisms may contribute to invasion and immune evasion of aEPEC in systemic infections, hence survival of aEPEC in the gut epithelial cells may lead to high morbidity. In this regard, Mercado et al. have demonstrated the detection of the complete PAI O122 associated with potential pathogenic strains of aEPEC [42]. However, further studies are needed to validate the findings.

Rehydration and treatment with oral zinc are the main modalities of management of EPEC diarrhoea in children. Antibiotics are not indicated in most cases because of its self-limiting course, the possibility of drug resistance, and the risk of antibiotic-associated adverse reactions [43]. Treatment with ciprofloxacin, azithromycin or 3rd generation cephalosporins may be indicated only when there are findings suggestive of associated systemic sepsis or urinary tract infection. Documented literature states that the drug resistance pattern of EPEC reflects a high rate of acquisition of the resistance due to aggressive usage of antibiotics [44]. In our study the isolates were resistant to cefotaxime (67%), gentamicin (31%) followed by nalidixic acid (21%) and piperacillin/tazobactam, denoting the existence of drug resistant EPEC in gut of children. It is also a reflection of the hurdle free ‘to and fro’ travel of the plasmids when an opportunity is provided. The ESBL and metallo β-lactamases(MBL) producing EPEC strains carry antibiotic resistance genes for ESBL (TEM, SHV, CTX, OXA) and MBL (NDM, IMP, VIM), respectively. They may act as important source of transfer of these resistance genes to other pathogens, and hence may work as a chief source of resistance in EPEC [45]. Therefore, the identification of DEC pathotypes becomes significantly important since the administration of antibiotics can increase the chance of transmission of not only resistance plasmids but virulence genes as well. Though, small number of investigated and identified EPEC isolates in our study may limit the generalizability of the findings, examination of a larger number of isolates needs to be undertaken in order to further elucidate these inferences.

Conclusions
Higher prevalence of EPEC with a higher incidence of aEPEC than tEPEC demonstrates a changing trend and the emergence of aEPEC as a dominant pathogen, compared to tEPEC, in east Delhi region of Northern India. Adaptive advantages and additional pathogenic mechanisms of aEPEC may contribute to this shift which needs further investigation.

Funding
This work was supported by the intramural funding from the University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi, India provided to Dr. Shukla Das.

Ethics approval and informed consent
The study was reviewed and approved by the Institutional Ethical Committee-Human Research of the University College of Medical Sciences & Guru Teg Bahadur Hospital, Delhi, India. All stool samples from the children were collected after obtaining written informed consent from the parents/guardians.

Conflicts of interest
The authors declare that they have no conflicts of interest.

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