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Prevalence of Enteropathogenic *Escherichia coli* (EPEC) in Adult Diarrhea Cases and their Antibiotic Susceptibility Pattern

S. Mariyam Sunaifa[1], Somdatta Roy[1] and B. Dhanashree[1*]

[1]Department of Microbiology, Kasturba Medical College, Manipal University, Mangalore, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author BD, designed the study, analyzed the results and critically evaluated & corrected the manuscript. Author SMS performed the experiment, wrote the protocol and wrote the first draft of the manuscript. Author SR managed the statistical analysis, literature searches & helped in writing the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Diarrheagenic *Escherichia coli* (DEC), an important etiologic agent of diarrhea is a major public health problem in developing countries. Relatively few studies have reported the role of enteropathogenic *E. coli* (EPEC) as etiological agent of adult diarrhea.
Objective: To know the prevalence of EPEC in adults and to know their antimicrobial susceptibility patterns.
Methods: Diarrheagenic stool samples (n=300), received at the department of Microbiology, Kasturba Medical College hospital, Mangalore, were cultured to isolate *E. coli* and other intestinal pathogens. Biochemically identified *E. coli* isolates were further characterized by polymerase chain reaction (PCR). Moreover, all the stool samples were subjected directly to PCR. Antibiotic susceptibility for EPEC was done by Kirby Bauer's disk diffusion method.
Results: Of the 300 stool samples processed, 61 samples showed the growth of *E. coli.* Four

**Corresponding author: E-mail: dbiranthabail@yahoo.co.in;*

samples had grown *Shigella flexneri*, three were *Vibrio cholerae* and One was *Aeromonas hydrophila*. Among the *E. coli* isolates characterized by PCR, four were typical EPEC, and atypical EPEC and one isolate was found to be Enterotoxigenic *E. coli* (ETEC). PCR performed directly on stool samples also yielded the same result. Antibiotic susceptibility testing revealed 42% of the *E. coli* other than DEC to be extended spectrum beta lactamase (ESBL) producers. However, one of the atypical EPEC was an ESBL producer.

Conclusions: In this study DEC, including EPEC types I and II, was found in a number of adult diarrheagenic stool samples and could be a possible cause of diarrhea in these patients. our study highlights the importance of PCR to differentiate atypical and typical EPEC. Presence of ESBL in commensal *E. coli* is a concern. Further characterization of these isolates from diarrheagenic individual and healthy controls is necessary to know their epidemiological significance.

Keywords: Antimicrobial susceptibility; enteropathogenic E. coli; PCR; stool sample.

ABBREVIATIONS

Enteropathogenic E. coli (EPEC)
Enteroaggregative E. coli (EAEC)
Enterotoxigenic E. coli (ETEC)
Enteroinvasive E. coli (EIEC)
Shiga toxin producing E. coli (STEC)
Diffusely adherent E. coli (DAEC)
Diarrhoeagenic E. coli (DEC)
Polymerase Chain Reaction (PCR)
Extended spectrum beta lactamase (ESBL)
Red blood corpuscles (RBC)

1. INTRODUCTION

Escherichia coli is one of the most important members of the family *Enterobacteriaceae*. They are the commonest cause of infections of the urinary tract and central nervous system [1,2]. Six categories of *E. coli* associated with diarrhea in several epidemiological studies are Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC),Shiga toxin producing *E. coli* (STEC) and diffusely adherent *E. coli* (DAEC) [3,4].

EPEC enteritis is common in communities with poor hygiene where sporadic cases and frequent out breaks occur in community as well as in institutions [5]. Importance of EPEC as a cause of enteritis in adults is difficult to evaluate due to two reasons. Firstly, adults may have antibodies in their serum due to childhood infection. Hence may get only subclinical infection and may not show symptoms. Secondly, many of the clinical laboratories in India consider *E. coli* isolates of stool as commensal and do not characterize them further. In a recent study from western Iran 47.5% of the diarrheagenic *E. coli* from adult patients were found to be EPEC [6,7]. However, literature search has not revealed data on the prevalence of EPEC among adults in southern India. Hence, an attempt was made to directly detect *E. coli* by PCR, and isolate them by culture from diarrhoeagenic stool. Further, the *E. coli* isolates were characterized by PCR and their antibiotic susceptibility pattern was studied.

2. MATERIAS AND METHODS

2.1 Specimen Collection

Over a period of one year (January to December 2012), diarrheagenic stool samples (n= 300) received at the Department of Microbiology, Kasturba Medical College Hospital, Mangalore, from adult patients who were more than18 years of age, were included in the study by following random sampling method. Individuals who were less than 18 years of age and those adults with diarrhea who were on antibiotic treatment were excluded from the study. This study was approved by the institutional ethics committee. Stool samples were transported at room temperature (25-30°C) and processed within 30 minutes of their receipt.

2.2 Microscopic Examination

Stool samples were initially screened microscopically for pus cells, red blood corpuscles (RBC)'s, ova and cysts of parasites. Two to three loops of liquid stool was placed on a clean glass slide and mixed with a drop of saline, covered with a cover slip and observed under low power objective and high power objectives to examine for pus cells, RBCs and trophozoites. Two to three loops of liquid stool was mixed with a drop of iodine solution on a glass slide, covered with a coverslip and observed under low power objective and high power objectives to detect ova and cysts of parasites.

2.3 Isolation of Enteric Pathogens

Stool samples were cultured on Sorbitol MacConkey's agar (SMAC), and MacConkey's agar. Enrichment culture was done by inoculation into selenite F broth (SFB) and alkaline peptone water (APW) and were incubated at 37°C for 6-8 hr. Enrichment broths were subcultured on Deoxycholate Citrate agar (DCA) and Thiosulphate Citrate Bile salt Sucrose agar (TCBS) respectively. All the culture plates were incubated at 37°C for 18hr. Both sorbitol fermenting and non-fermenting colonies (n=5) from SMAC and lactose fermenting and non-fermenting colonies (n=5) from MacConkey's agar, lactose non-fermenting colonies from DCA, and sucrose fermenting colonies from TCBS were picked and identified by standard biochemical tests [8]. The tests included catalase, oxidase, fermentation of lactose, glucose and sucrose using triple sugar iron agar, decarboxylation of lysine using lysine iron agar, production of indole, methyl red test, voges proskauer and utilization of citrate. The enteric pathogenic bacterial isolates and *E. coli* isolates were preserved at -20°C in 20% glycerol broth for further characterization. Apart from the bacterial isolates seven stool samples showed the growth of *Candida* spp. on MacConkeys agar plates which were not speciated further.

2.4 Antimicrobial Susceptibility Testing

Antibiotic susceptibility test was performed by Kirby Bauer's disk diffusion method. Briefly, biochemically confirmed *E. coli* isolates were grown in Muller Hinton broth for 6 hr at 37ºC. Turbidity was adjusted to 0.5 Mc Farland standard and Muller Hinton agar plates were seeded with culture. Different antibiotics (Himedia laboratories Ltd, Mumbai, India) like ampicillin, ceftazidime, ceftazidime / clavulanicacid, cefotaxime, ciprofloxacin, cefuroxime, cefoxitin and gentamicin were placed on the medium. Antibiotic sensitivity plates were incubated at 37°C for 24hr. *E. coli* ATCC25922 was used as quality control strain. Zones of clearing around the disks were measured and compared with Clinical and Laboratory Standard Institute (CLSI) standards and interpreted as either sensitive, resistant or intermediate [9].

Isolates were tested for extended spectrum beta lactamase (ESBL) production by the combination disk method using ceftazidime (30 µg) and ceftazidime /clavulanic acid (10 µg). A ≥5 mm increase in diameter of the inhibition zone of the cephalosporin-plus-clavulanate disc when compared to the cephalosporin disc alone were interpreted as phenotypic evidence of ESBL production. *Klebsiella pneumonia* ATCC 700603 was used as an ESBL producing control and *E. coli* ATCC 25922 as a negative control [9].

2.5 DNA Extraction and PCR

DNA from all stool sample was extracted by using QIA amp stool kit (Genetix Asia Pvt.Ltd., Bangalore) following the manufacturer's instructions. PCR was performed on all the stool samples as it is highly sensitive and specific test. However PCR was done on all the 61 biochemically confirmed *E. coli* to categorize them in to various DEC. DNA from *E. coli* Isolates were extracted by boiling method. Briefly, three *E. coli* colonies were inoculated into 200 µL of distilled water. Boiled for 15 min at 95°C in a dry bath, centrifuged at 12000 g for 5 min.1 µL supernatant is used as DNA in PCR.

Primers given in Table 1 were used for the detection of the virulence genes of *E. coli* based on the previously published reports [10]. *E. coli* reference strain EDL 933 was used as positive control for EPEC and STEC. Reference strain E2348/69, were used as positive control for ETEC PCR reactions. PCR was carried out for 35 cycles in the thermo cycler. The reaction conditions were: initial denaturation at 95°C for 5 min, denaturation at 95°C for 1 min, primer annealing at 60°C for 1.5 min, extension at 72°C for 1.5 min and final extension at 72°C for 5 min. Amplified products were separated by using 2% agarose gel, stained with ethidium bromide and photographed using gel documentation system [11].

3. RESULTS AND DISCUSSION

Of the 300 samples processed, 159 were without pus cells and RBC and did not yield any bacterial pathogens by culture or PCR. Of the 141 stool samples with pus cells, 61samples showed the growth of biochemically confirmed *E. coli* isolates, four samples *S. flexneri,* three samples *V. cholerae*, one sample Aeromonas hydrophila and seven samples had the growth of Candida spp. The remaining 65 samples with pus cells did not yield any bacterial pathogens. Parasitic ova and cysts were not seen in the stool samples screened.

Table 1. Primers used in PCR for the detection of diarrheagenic *E. coli* strains [10]

E. coli strains	Locus	Primers	Amplicon size (bp)
ETEC	*lt*	F:5'GGC GAC AGA TTA TAC CGT GC3' R:5' CGG TCT CTA TAT TCC CTG ET3'	450
ETEC	*st*	F:5'ATT TTT CTT TCT GTA TTG TCT T3' R:5'CAC CCG GTA CAA GCA GGA TT3	190
EPEC	*bfp*A	F:5' AAT GGT GCT TGC GCT TGC TGC3' R:5' GCC GCT TTA TCC AAC CTG GTA3	324
EPEC	*eae*A	F:5'GAC CCG GCA CAA GCA TAA GC3' R:5 CCA CCT GCA ACA AGA GG3	384
STEC	EHEC*hly*A	F:5' ACG ATG TGG TTT ATT CTG GA3' R:5'CTT CAC GTC ACC ATA CAT AT3'	166
EIEC	*ial*	F:5' GGT ATG AGT CCA3' R:5' GGA GGC CAA TTA TTT CC3	650

PCR was performed on DNA extracts of 61 biochemically confirmed *E. coli* isolates and also on all 300 stool samples to detect the different virulence genes of diarrheagenic *E. coli* (DEC). Four *E. coli* isolates were positive for both eaeA and bfpA genes. One *E. coli* was positive for both st and lt genes, two were positive for only st genes and four isolates were Positive for only eaeA gene Fig. 1. Rest of the 50 *E. coli* isolates were negative for all the DEC genes tested. Various bacterial pathogens isolated from stool samples are shown in Table 2.

Out of the 300 stool samples directly tested by PCR,148 were negative for all the DEC genes. Four stool samples were positive for both eaeA and bfpA, another four were positive for only eaeA gene, two were positive for only st gene and only one was positive for both st and lt genes as shown Table 3.

Most of the *E. coli* strains isolated showed resistance to the antibiotic tested. None of the strains was 100% sensitive to the antibiotic tested. Antibiotic susceptibility pattern of biochemically confirmed *E. coli* isolates is shown in Table 4.

The prevalence and epidemiological significance of *E. coli* category isolated from stool sample varies with the geographical area. In the present study, various pathogens were isolated from diarrhoeagenic stool samples of adults in addition to EPEC (n=04) atypical EPEC (n=04) and ETEC (n=01). Generally, EPEC are of two types. Type I or typical EPEC are those, which are positive for both *eae*A and *bfp*A, genes. Type II or atypical EPCE are those which are positive for only *eaeA* gene and lack especially *bfpA* gene and other DEC genes [12,13]. In the study, we have isolated both typical and atypical EPEC as sole pathogens from stool samples and detected them in stool samples directly by PCR.

Table 2. Microorganisms isolated from diarrheagenic stool samples

Name of theorganism	Number of isolates
Candida species	7
Shigella flexneri	4
Vibrio cholerae	3
Aeromonas hydrophila	1
EPEC	4
Atypical *EPEC*	4
ETEC	1
E. coli with only *st* genes	2
Commensal *E. coli*	50

Studies from India and abroad have reported an increasing trend in the isolation of atypical EPEC than typical EPEC from childhood diarrhea [1,14-17]. It is true in the present study concentrated on adult diarrhea patients, where 6.6% of the isolates were typical and atypical EPEC. Hence these *eaeA* positive atypical EPEC requires further study with regard to their virulence and epidemiologic significance and serotyping. Further, only1.6% of the isolates were ETEC (positive for both *st* and *lt* genes) which is found to be lower than the previously reported data from India [2,7], which was mainly focused on pediatric age group, unlike the current study. However, two *E. coli* isolates were positive for only *st* genes and negative for *lt* genes. These *st* gene positive *E. coli* isolates needs further characterization for expression of colonization factor genes of ETEC. Recently, EPEC has been reported to be a commonly identified DEC strain in adult diarrhea cases in Iran [6]. However, prevalence of EPEC among adult diarrhea cases in India is not available to compare with our data.

Table 3. Detection of virulence of genes of diarrheagenic *E. coli* by PCR

Samples tested	Virulence genes detected			
	eaeA	***bfpA***	***st***	***lt***
Direct stool samples (n=61)	8	4	3	1
E. coli isolates (n=61)	8	4	3	1

Table 4. Antibiotics susceptibility pattern of *E. coli isolates* (n=61)

Antibiotic tested (µg)	Commensal *E. coli* (n=50)			Diarrheagenic *E. coli* (n=11)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Ampicillin(10)	0(0)	0(0)	50(100)	8(72.7)	0	3(27.3)
Ceftazidime(30)	2(4.0)	0(0)	48(96.0)	10(90.9)	0	1(9.1)
Cephotaxime(30)	1(2.0)	0(0)	49(98.0)	11(100)	0	0(0)
Ciprofloxacin(5)	3(6.0)	1(2.0)	46(92.0)	10(90.9)	0	1(9.1)
Cefuroxime(30)	2(4.0)	0(0)	48(96.0)	10(90.9)	0	1(9.1)
Cephoxitin(30)	2(4.0)	0(0)	48(96.0)	11(100)	0	0(0)
Gentamicin(10)	13(26.0)	0(0)	37(74.0)	11(100)	0	0(0)
Ceftazidime clavulanic acid (30)	29(58.0)	0(0)	21(42.0)	10(90.9)	0	1(9.1)

**S: sensitive R: Resistant I: Intermediate*

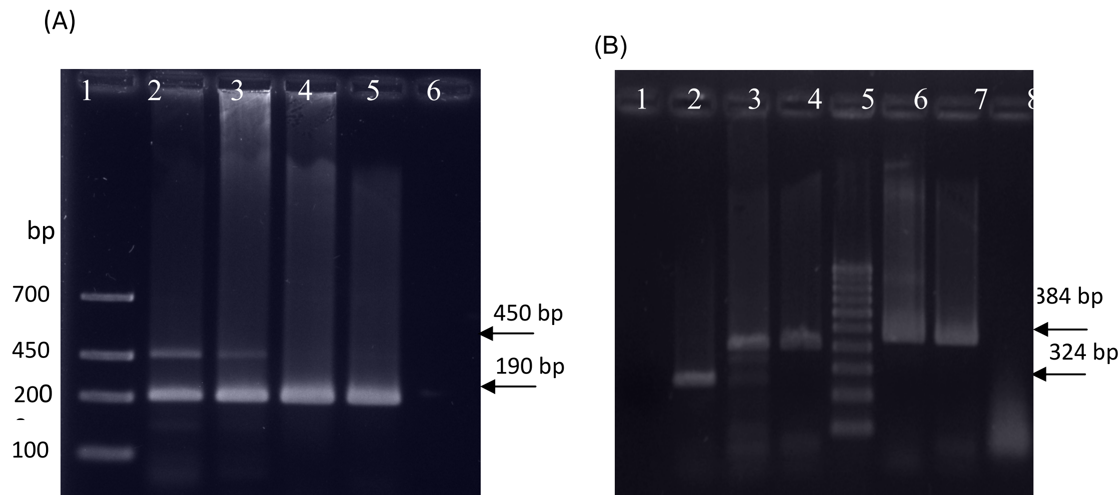

Fig. 1. Agarose gel picture showing the amplification product of PCR performed on *E. coli* isolate for the detection of ETEC genes (A) and EPEC genes (B)

(A) Lanes 1: Molecular weight marker; 2: ETEC positive control; 3: isolate positive for st< 4 &5: isolates positive for st; 6: Negative control (B) Lanes 1 and 8: Negative control of bfpA & eaeA; 2: bfpA positive isolate; 3: bfpA & eaeA positive isolate; 4 & 6: eaeA Positive isolates; 5: Molecular weight marker (100 bp ladder);7: eaeA positive control.

E. coli isolates, negative for all the DEC genes (n=50) by PCR were considered as commensal *E. coli* or the normal intestinal flora. However, if only routine biochemical identification was performed on *E. coli* isolates from stool, DEC would have been missed or would have been considered as normal intestinal flora. Hence this study highlights the importance of a sensitive, specific and rapid test like PCR to characterize the virulence genes of all *E. coli* isolates from stool in order to differentiate DEC from commensals.

Therapeutic options vary depending on the DEC strain isolated. DEC strains like ETEC needs to be treated with antibiotics, while STEC should not be treated with antibiotics. Hence rapid differentiation of DEC and knowing their antibiotic susceptibility pattern plays an important role in patient management. In our study, one of the atypical EPEC strains was ESBL producer is interesting to note. Typical EPEC and ETEC were sensitive to all the antibiotics tested (Table 4). 68.8% of the commensal *E. coli* were found to be ESBL producers. Increasing

antimicrobial resistance seen in commensal *E. coli* isolates could be due to indiscriminate use of antimicrobials in clinical practice and sale of antibiotics across the counter. High prevalence of antimicrobial resistance among EPEC strains was documented in different parts of the world [14,18,19]. In this study, resistance was seen more commonly in typical EPEC than in atypical strains which is in agreement with the earlier findings. However one of the atypical EPEC was ESBL producer. Hence rapid detection and differentiation of DEC from commensal *E. coli* from stool samples by PCR plays an important role in patient management.

4. CONCLUSION

Results of our study highlights the importance of characterization of all *E. coli* strains isolated from diarrhoeagenic stool samples by PCR. If EPEC are detected in stool samples of adults suffering from diarrhoea it should not be ignored. Further, direct detection of DEC virulence genes in stool samples by PCR would save time and also help in fast patient management. Further studies are necessary to characterize large number of typical and atypical *E. coli* isolates from diarrhoeagenic stool samples to know their pathogenic potential.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the Institutional ethics committee of Kasturba Medical College Mangalore, and have therefore been performed in accordance with the ethical standards.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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