

RESEARCH ARTICLE

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Characterization of Enterotoxigenic and Enterohemorrhagic *Escherichia coli* in Paediatric Patients

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Abstract

In developing countries diarrheal illness are causing severe health problem in children up to five years of age group and it also results in causing the morbidity and mortality. Worldwide among all the bacterial pathogens diarrheagenic *Escherichia coli* is responsible for epidemic and endemic diarrhoea. *E. coli* rarely cause disease in healthy individuals and most of *E. coli* strains live harmlessly in the intestines. The objective of the study was to detect six categories of DEC in one set of Multiplex PCR reactions. Total number of 155 in and outdoor children stool sample with complain of diarrhoea were collected in Rama Medical College, Kanpur, India. Target genes used for amplification by Multiplex PCR were *eaeA* and *bfpA* for EPEC, *elt* and *stx* for ETEC, *CVD432* for EAEC and *hlyA* for EHEC. Out of 155 isolates examined, 71 were *E. coli* isolates, out of 71 isolates 28 were ETEC, 02 were EHEC. *elt* gene of ETEC and *hlyA* gene of EHEC diarrheagenic *E. coli* were detected. Antibiotic sensitivity tests for diarrheagenic strains were done by Kirby Bauer Method. Antibiotics used in the study were Ampicillin, Cotrimoxazole, Ciprofloxacin, Norflox, Nalidixic acid, Cefotaxime, Chloramphenicol, Imipenem, Azithromycin, Gentamicin. In most cases ETEC and EHEC diarrheagenic isolates were found resistant to Ampicillin, Gentamicin, Cotrimoxazole and Azithromycin. Multiplex PCR, a rapid method for detecting virulent genes of DEC at a time and it also provide a platform in understanding the diarrheal diseases in a more improved manner.

Keywords: *E. coli*, morbidity, mortality, Antibiotic sensitivity, ETEC, EHEC, endemic

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INTRODUCTION

Globally diarrheal diseases in low- to middle- income countries and children under five are main cause of morbidity and mortality and also results in 0.5 million deaths¹. Diarrhoea is caused by various numbers of parasites, bacteria and viruses². *E. coli* is facultative anaerobic and non-pathogenic member of the human gastrointestinal microbiota³. Association with certain serotypes the diarrheagenic strains of *E. coli* is divided into five main categories: enterotoxigenic *E. coli*, enterohemorrhagic *E. coli*, enteropathogenic *E. coli*, enteroaggregative *E. coli* and enteroinvasive *E. coli* and are based on specific virulence determinants, distinct epidemiological and clinical features⁴. *E. coli* is normal flora and plays pathogenic role in human host. Worldwide Diarrheagenic *E. coli* (DEC) is reported as one of the main causes of gastrointestinal disorders⁵⁻⁸. Enterotoxigenic *E. coli* (ETEC) causes traveller's diarrhoea and in population and it is a significant pathogenic form of *E. coli*⁹⁻¹¹. Amplification of two marker genes *elt* and *stla* that encodes heat stable and heat labile secretory enterotoxins confirms ETEC in the stool specimen.

Gastrointestinal infections is caused by Enterohemorrhagic *E. coli* (EHEC), a subgroup of Shiga toxin- producing *E. coli* and it secretes toxins so EHEC colonizes large intestine which may lead to severe complications like (HUS)haemolytic uremic syndrome¹².

Polymerase chain reaction (PCR) provides a rapid, profitable and sensitive method in identification of enteric pathogen in developing countries. This study was designed to calculate the preponderance of DEC in paediatric group below 5 years of age suffering from diarrhoea by multiplex PCR as they are present as commensal in non-diarrhoeal stool samples of healthy children and to determine the aetiology of infantile diarrhoea by examining the pathogenic *E. coli* strains acidity property.

MATERIALS AND METHODS

Sampling

155 diarrheal patients aged between 0 to 5 years with ailment of abdominal pain, vomiting, fever, three or more loose stools/day and mucus or blood in stool, admitted or opd to Rama Medical College Hospital, Kanpur were collected.

Identification and isolation of *E. coli* Isolates

All faecal specimens were first processed for wet mount for screening of parasites as an exclusion criterion. Then gram staining was done for screening of gram negative bacteria and presence of pus cells. Gram negative bacteria cultured on Eosin Methylene Blue (EMB) appear as green with black metallic sheen colony, are again sub cultured on fresh EMB agar plates to obtain presumptive *E. coli* isolates. These presumptive *E. coli* isolates were confirmed by the confirmatory biochemical test (IMViC) for *E. coli*.

Isolation of *E. coli* O157:H7

The confirmed *E. coli* isolates were sub cultured on Sorbital MacConkey Agar plates (SMAC), and its colonies appears colourless on SMAC were presumptive method of *E. coli* O157.

Molecular Characterization

All gram negative lactose fermenting growths of *E. coli* were confirmed by molecular characterisation method. DNA of all lactose fermenting growth of *E. coli* strains was extracted by Qiazen DNeasy Ultra clean microbial kit.

E. coli colonies for the virulence genes were confirmed by Multiplex PCR. The primers (Table 1) selected for six different virulence genes (*elt*, *eaeA*, *stla*, *bfp*, *CVD432* and *hlyA*) concurrently in a single reaction. EHEC and ETEC were identified by the appearance of the *elt* and *hlyA* genes, which are present only in ETEC and EHEC. Total volume of 25µl in a reaction mixture is performed by adding 12.5µl PCR master mix 2x, manufactured by Quantitect multiplex PCR kit containing 400 mM deoxy-nucleoside triphosphates, 0.05 U/µlTaq DNA polymerase, 4 mM MgCl₂, 5.5µl sterile double distilled water, 1µl from each primers, and 5µl DNA template. Cycling parameter used are as follows: 94°C for 4 min for initial denaturation of the DNA, then 30 cycles for 30 sec at 94°C, 30sec at 59°C, 1.30 min at 72°C, and lastly final single prolonged extension at 72°C for 10 min for *elt* genes, and 3 min at 95°C, then 35 cycles for 30sec at 94°C, 30 sec at 55°C, 30 sec at 72°C, and finally a single prolonged elongation at 72°C for 10 min. The amplified product were visualized by gel electrophoresis in 1.5% agarose gel containing ethidium bromide for 45 min at 100 V and then it is visualized under UV light of gel documentation system.

Antibiotic Sensitivity testing

Antibiotic sensitivity test for the isolates that yielded positive results in the Multiplex PCR assay were cultured on Mueller-Hinton agar by the Kirby- Bauer method, following Clinical and Laboratory Standard Institute (CLSI)¹³. Ten antibiotic discs (Himedia) of different classes that are generally used in medical diagnostic laboratories were selected. The discs used were Nalidixic acid (NA, 30µg), gentamicin (G, 10µg), ampicillin (AMP, 10µg), Cefotaxime (CTX, 10µg), Cotrimoxazole (SXT, 25µg), Imipenem (IPM, 10mcg), Azithromycin (AZM, 15µg), Norflox (NOR, 10µg), ciprofloxacin (CIP, 5µg), chloramphenicol (C, 30µg).

Ethical consideration

Institutional ethics review board of Rama Medical College, Kanpur approved the study.

Before enrollment into the study, written consent was taken from parents/guardians of the children.

RESULTS

Out of 71 *E. coli* isolates (five-six colonies from each stool sample) which were identified by biochemical tests (Table 1), 30 isolates were identified as diarrheagenic *E. coli* by Multiplex PCR. Out of which 28 (39.4%) were ETEC (elt positive) and 02(2.8%) were EHEC (hlyA positive) as shown in Fig. 1, 2 and 3. Gender distribution was 17(60.71%) female and 11(39.28%) male in case of ETEC strains and 0 (00%) male and 2 (100%) female in case EHEC strains shown in Fig. 4 and 5. The sex distribution was 17 (60.71%) male and 11 (39.28%) female in case of ETEC strains.

Antimicrobial susceptibility testingThe result of antibiotic sensitivity testing of ten

Table 1. Biochemical test applied for screening of *E. coli* in paediatric patients

No.	Indole	MR	TSI	Citrate	Urease	Mannitol	Total (N)	Positive
1	Positive	Positive	A/A	Negative	Negative	Positive	155	71

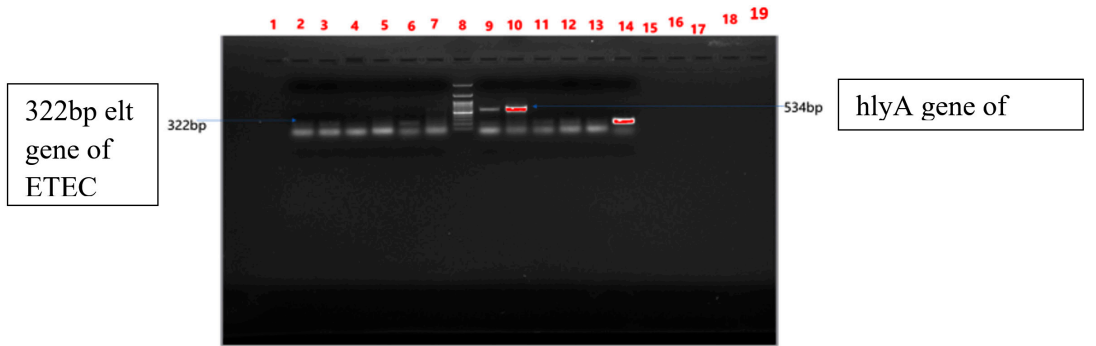


Fig. 1. Multiplex PCR of pediatric samples. Lane 2-7 and 11-14 ETEC (amplicon size 322bp) and Lane 9-10 EHEC (amplicon size534bp) Lane 8: Ladder (100bp -1000bp)

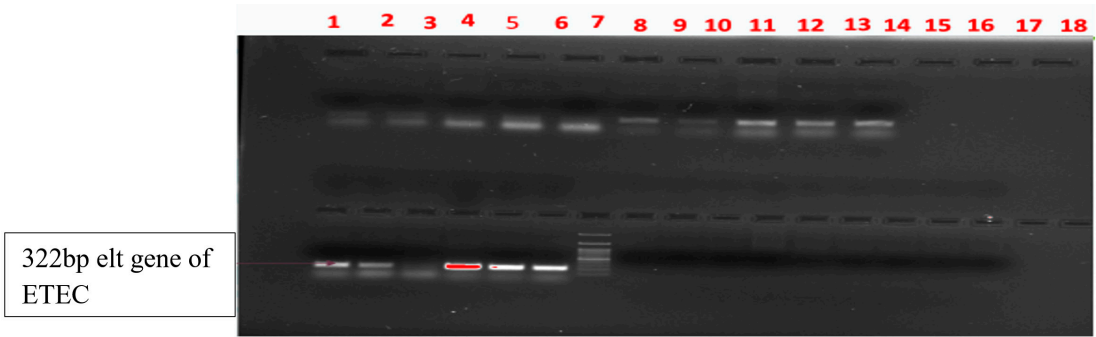


Fig. 2. Lane: 1, 2, 4, 5 and 6 contains elt gene of 322bp of ETEC gene and Lane 7 contain ladder (100bp-1000bp)

antibiotics for 71 *E. coli* isolates isolated from diarrheal patients are shown in Fig. 6 and 7. The most common sensitive profile of antibiotics found were Imipenem, Nalidixic acid and ciprofloxacin. Susceptible rate of 100% Imipenem was found to be the most effective antibiotic in 28 strains of ETEC strains (Fig. 8). Of the 2 EHEC strains tested, cefotaxime and chloramphenicol was found to be most sensitive with susceptible rate of 100% (Fig. 9).

DISCUSSION

Worldwide diarrheagenic *E. coli* strains are found to be the most important paediatric enteropathogens¹⁴. Bacterial and parasitic pathogens with rotavirus, viral, diarrheagenic *E. coli*, *Shigella spp.* and *Salmonella spp.* are the cause of diarrhoea in developing countries¹⁵. In the current research work blood was found in diarrheal patients with the EHEC patho type, but two of them belonged to the O157:H7 serotype.

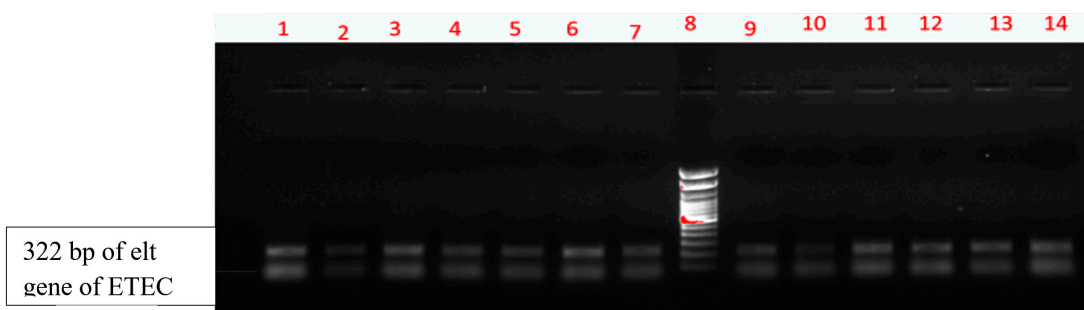


Fig. 3. Lane: 1-7 and 9-14(13samples) contains elt gene of 322bp of ETEC gene and Lane 8: Ladder (100bp-1000bp)

Gender Distribution (N=28)

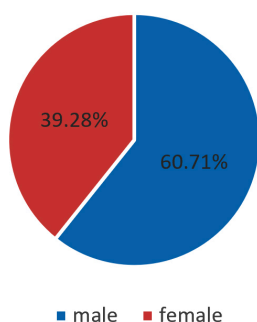


Fig. 4. Gender Distribution (N=28) ETEC *E. coli*

Gender Distribution (N=2)

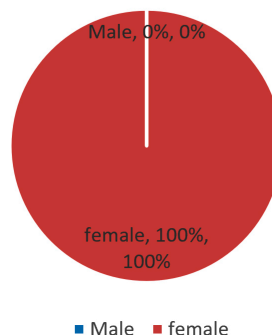


Fig. 5. Gender Distribution (N=2) of EHEC *E. coli*

Sensitive

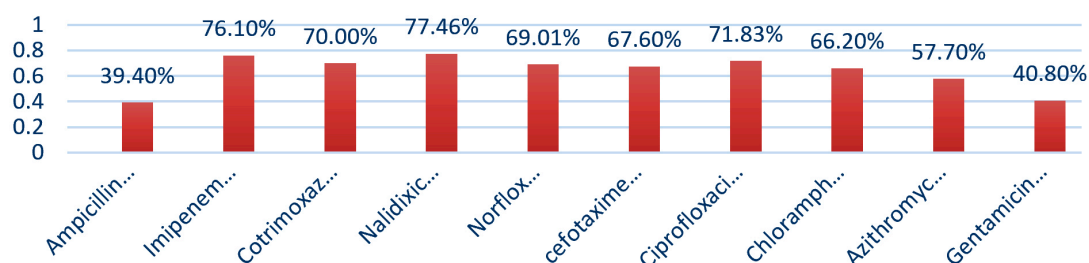


Fig. 6. Antibiotic sensitivity data of 71 *E. coli* isolates

In previous studies it is found that EHEC was less frequently isolated than other of diarrheagenic *E. coli*¹⁶. Concurrence with the low profile of EHEC infection was noted in the current study. Vilchez et al. found few EHEC strains in diarrheal patient in developing countries¹⁷. In the present study,

diarrhoea causing *E. coli* has the highest rate of ETEC (39.4%) strains. ETEC strains were detected significantly more (60.71%) in adult man with diarrhoea. In some studies in developing countries it was observed that EHEC was less frequently isolated than ETEC or EPEC strains¹⁸. The most important pathogen responsible for traveller's diarrhoea is ETEC and it causes approx 700,000 childhood deaths per year, mostly in paediatric group¹⁹. Widespread use of antibiotics is the cause of high incidence of antibiotic-resistant in isolates of DEC. Diverse resistance construction to usual antibiotic results is due to transfer of resistance genes between species. The ecological and pathogenic characters of bacterial species are changed by these transfers²⁰. In our study, the rate of antibiotic-resistant is high in isolates of EHEC was seen to Ampicillin and Cotrimoxazole (100%). The drug sensitivity pattern of our isolates demonstrated a good response to Norflox and Ciprofloxacin (100%). There are consistent reports from other authors that frequent resistance to common antibiotics such as tetracycline, ampicillin and trimethoprim-sulfamethoxazole was detected in ETEC strains²¹⁻²⁶.

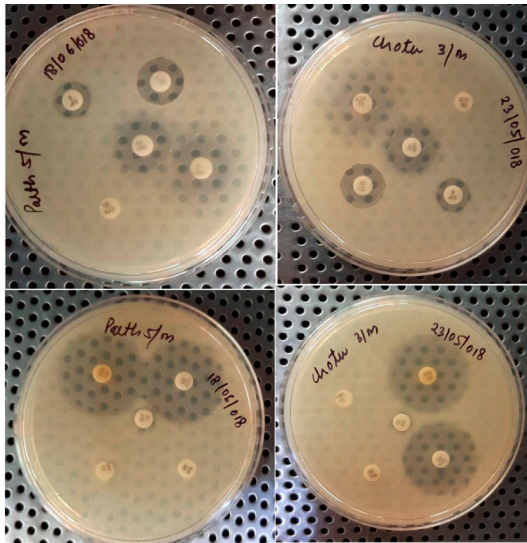


Fig. 7. Antibiotic Sensitivity test depicting zone of inhibition of ten antibiotics used

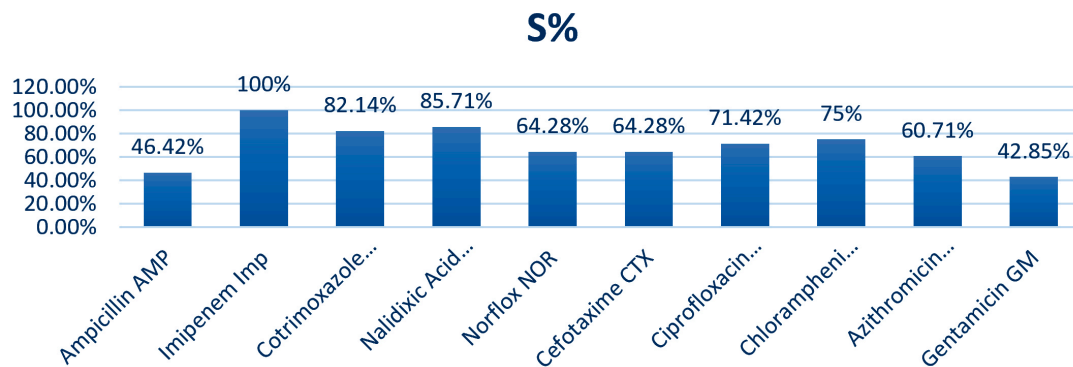


Fig. 8. Antimicrobial sensitivity of ETEC Strain of *E. coli* (N=28)

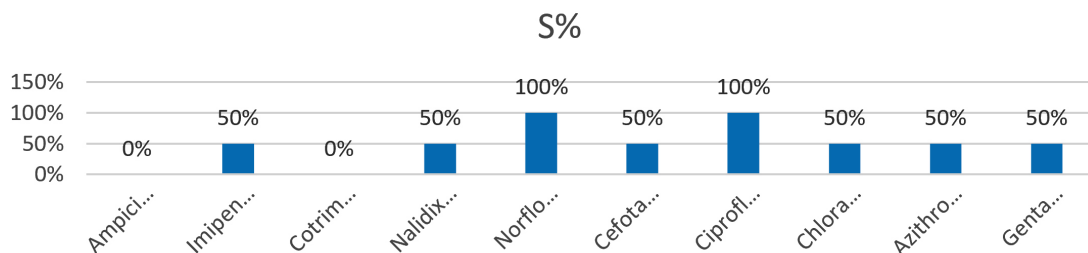


Fig. 9. Antimicrobial sensitivity of 2 EHEC isolates

CONCLUSION

The detection of ETEC and EHEC in this region suggested that this strain is main common source of diarrhoea among paediatric age group. Education on diarrheal infections management should be taught to the parents/guardians of the children affecting with diarrhoea as well as the implementation of more stringent policies governing the availability of antibiotics should be given. Heavy clinical use of antibiotics, and have a low cost leads to high-level resistance to antibiotics. The indiscriminate use of antibiotics needs to be avoided and the guidelines should be made for proper use of antibiotics for treatment of diarrhoea in the region needs to be established.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None.

AUTHORS' CONTRIBUTION

All authors listed have made equal substantial and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY

All datasets analysed in the study are included in the manuscript and presented as tables and figures.

ETHICS STATEMENT

Study is ethically approved by Rama University, Kanpur U.P. with reference no. 90 Res/Reg/RUK/2017.

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