

Original Research Article

<http://dx.doi.org/10.20546/ijcmas.2016.503.056>

Molecular Characterization of Diarrhegenic *Escherichia coli* from Tiruchirappalli District, Tamilnadu, India

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ABSTRACT

Keywords

Diarrhegenic
Escherichia coli
(DEC),
Molecular
characterization.

Article Info

Accepted:

18 February 2016

Available Online:

10, March 2016

Diarrhegenic *Escherichia coli* (DEC) are important enteric pathogens that cause a wide variety of gastrointestinal diseases, particularly in children. *Escherichia coli* isolates cultured from 185 diarrheal stool samples obtained from in and around Tiruchirappalli District, Tamilnadu, India. The samples were screened by polymerase chain reaction (PCR) for genes characteristic of enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC). The DEC were detected only one ETEC gene were detected, were EAEC; EHEC, and EIEC were not detected. The EAEC isolates were also tested for eight genes associated with virulence using PCR.

Introduction

Although part of the normal gut flora, *Escherichia coli* strains are also important intestinal pathogens. Diarrhegenic *E. coli* (DEC) cause a wide variety of gastrointestinal diseases, particularly among children in developing countries, resulting in significant morbidity and mortality (Moyo, 2007; Khairun, 2007; Brandal, 2007; Nguyen, 2005; Rajendran, 2010; Franzolin, 2005). The DEC are differentiated into at least five pathotypes, according to their pathogenic mechanisms,

that include enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC) (also known as Shiga-toxin producing *E. coli* or verocytotoxigenic *E. coli*) (Natoro, 2005; Adachi, 2002).

Materials and Methods

Clinical Specimens

During the period from April 2012 to

September 2014, 185 stool samples from children who were from 0-60 months of age were investigated in Tiruchirappalli Tamilnadu, India to determine the prevalence of diarrheagenic *E.coli* as the etiological agents of diarrhea (Moyo, 2007; Khairun, 2007; Brandal, 2007; Nguyen, 2005; Rajendran, 2010; Franzolin, 2005). This included 90 patients with and 95 children without diarrhea. Cases and controls were selected from patients attending the outpatient clinic of three different government hospitals after receiving permission from institutional ethical committee. Children were enrolled in the study if they had diarrhea characterized by the occurrence of three or more, loose watery stool or at least one bloody loose stool in a 24 hour period. Neither patients nor controls had been treated with antibiotics in the week preceding sampling. The stool samples were cultured on Mac Conkey agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India). As a rule about 5 lactose fermenting colonies presumed to be *E.coli* by colony morphology, were selected and submitted to biochemical tests. Biochemically confirmed *E.coli* isolates were stored at - 80°C in Trypticase soy broth supplemented with 20% glycerol for further procedures.

Bacterial Strains

Escherichia coli isolates were recovered from 143 diarrheal stool samples from the period of two years sample collection of age up to 12 years of age (> 90% were < 3 years of age). Stool samples were collected at Government hospitals in and around Tiruchirappalli district and outpatient clinics of the cities between April 2012 and March 2013 and the summer of 2014. After receiving informed consent from a parent or guardian, a clinical history for each patient was obtained. Histories were obtained through physical examination by medical doctors. Clinical symptoms, including fever,

vomiting, abdominal pain, and dehydration were recorded in a standard proforma. Stool specimens from enrolled children were collected using wide-mouthed sterile plastic containers and transported immediately to the microbiology laboratory for analysis within two hours of collection.

The reference strains were *E.coli* ATCC 43887 positive for *eaeA* and *bfpA* genes of EPEC; *E.coli* ATCC 35401 positive for *elt* and *stla* genes of ETEC; *E.coli* ATCC® 43893 positive for *ial* gene of EIEC. Local isolates of *E.coli* positive for CVD432 gene to detect EAEC and *hly* gene to detect EHEC were used for standardization of the multiplex PCR assays. *E.coli* isolated from children with and without diarrhea was subjected to the multiplex PCR assays described below to detect the presence of DEC.

Multiplex PCR Assays

The multiplex PCR assays were standardized for the detection of five types of DEC. The reference strains were cultured on Mac Conkey agar. A sweep of about five *E.coli* like colonies was used for PCR. The DNA was isolated from colonies by suspending the colonies in 50µl of deionized water. The suspension was boiled for 10 min at 95°C and centrifuged at 10,000 × g for 10 min. The supernatant was then used as the DNA template. The DNA templates were subjected to multiplex PCR with specific primers [Table 1], for the detection of the following virulence markers: *eaeA* for the structural gene of intimin of EPEC and EHEC, *bfpA* for the structural gene of the bundle forming pilus of EPEC, *hlyA* for the plasmid encoded enterohemolysin of EHEC, *elt* and *stla* for the enterotoxins of ETEC, *ial* for the invasion associated locus of the invasion plasmid found in EIEC, CVD432 for the nucleotide sequence of the EcoRI-Pst DNA fragment of EAEC. The

primers were selected on the basis of similar studies done earlier. The specificity of each primer was confirmed by monoplex PCR. The multiplex PCR assays were tested with several PCR cycling protocols.

The reaction mixture containing optimized protocol was carried out with a 50 µl mixture containing 10mM Tris-HCL (pH 8.3), 50mM KCL, 2.0 mM MgCl₂, a 2mM concentration of each deoxynucleoside triphosphate (Fermentas Inc, Maryland, USA), 2 U of Hot start Taq DNA polymerase (Fermentas Inc., Maryland, USA), 5 µl of the DNA template. 0.5 µM of each of the primers (Sigma-Aldrich Co., St. Louis, MO, USA) i.e. *elt* and *stlA* for ETEC isolates, CVD432 for EAEC isolates and *hlyA* and *eaeA* for EHEC isolates. The cycling conditions in Eppendorf's Mastercycler pro (Eppendorf India Limited Chennai, India) were as follows: 95°C for 1 min for one cycle followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1min and 72°C for 5 min. The protocol used was as explained above for Multiplex PCR except that the primers used were *eaeA* and *bfpA* for EPEC isolates, and *ial* for EIEC isolates. The thermo cycling conditions were as follows: 95°C for 1 min for one cycle followed by 35 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1min and 72°C for 5 min. The PCR products (10 µl) were analyzed by gel electrophoresis with 2.0% (W/V) agarose gels (HiMedia Laboratories Pvt. Ltd., Mumbai, India) in Trisborate- buffer (Fermentas Inc., Maryland, USA). The DNA bands were visualized and photographed under UV light after staining the gel with ethidium bromide (Fermentas Inc., Maryland, USA).

Sensitivity of Multiplex PCR Assays

To determine the detection limit of the multiplex PCR assays stool samples negative for diarrheagenic *E.coli* were spiked with a phosphate buffered saline

suspension of reference strains of EAEC and EIEC in serial 10 fold dilutions to give 10⁰ to 10⁸ CFU/ml (Moyo, 2007; Khairun, 2007; Brandal, 2007; Nguyen, 2005; Rajendran, 2010; Franzolin, 2005). Each serial dilution of the spiked stool sample was spread onto a Mac Conkey agar and incubated at 37°C. Multiplex PCR was performed with each dilution of spiked stool sample. The sensitivity of the assay was defined as the lowest concentration of DEC that yielded positive results for each dilution. The sensitivity of detection was 10³ CFU per assay for all target genes. The PCR assays were also used to detect DEC directly from fecal samples spiked with different concentrations of the reference strains of EIEC and EAEC. The reference strains were suspended in phosphate buffered saline at different concentrations. The diluted suspensions were used to spike 180 mg of stool specimen. The DNA was isolated from the spiked stool specimens by using QIAamp stool mini Kit (QIAGEN India Pvt. Ltd, New Delhi, India) and subjected to multiplex PCR assays. The detection limit by the multiplex PCR assays for both the reference strains was 10⁸ CFU.

Specificity of Multiplex PCR Assays

The specificity of the two multiplex PCR assays was determined by using standard reference and ATCC strains of DEC. The strains were subjected to both multiplex PCRs, and the results were compared with those obtained by monoplex PCR assays. Both multiplex PCR assays showed 100% specificity in identifying the reference strains as shown in. Non-specific bands were not visualized.

Results and Discussion

Detection and Identification of DEC Strains from Stool Sample

A total of 75 DEC was isolated from 125

stool samples from children with diarrhea. DEC was detected in 16 (16%) out of 95 stool samples from children without diarrhea. The prevalence of DEC in both groups was significantly different ($P < 0.05$). The prevalence was greatest for EAEC both

children with and without diarrhea. The PCR assays detected only ETEC isolates. EPEC and EHEC were not isolated from the stool samples without diarrhea. EAEC was not isolated from any of the children tested.

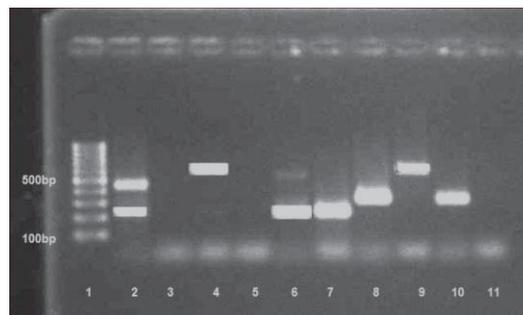
Table.1 PCR Primers used in the Multiplex PCR Assays for the Detection of Virulence Genes of Diarrheogenic *E.coli*

Reference strain	Primers (5' to 3')	Target gene	Product size (bp)
EPEC	TGATAAGCTGCAGTCGAATCC CTGAACCAGATCGTAACGGC	<i>eaeA</i>	229
	CACCGTTACCGCAGGTGTGA GTTGCCGCTTCAGCAGGAGT	<i>bfpA</i>	450
ETEC	CTCTATGTGCACACGGAGC CCATACTGATTGCCGCAAT	<i>elt</i>	322
	TCTTTCCCCTCTTTTAGTCAGTC CCGCACAGGCAGGATTAC	<i>Stla</i>	170
EIEC	CTGGTAGGTATGGTGAGG CCAGGCCAACAAATTATTTC	<i>ial</i>	320
EAEC	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	<i>CVD432</i>	630
EHEC	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	<i>hlyA</i>	534

Table.2 Diarrheogenic *E.coli* among Children with and without Diarrhea

Diarrheogenic <i>E.coli</i>	Positive	Negative
Enteroadherent <i>E.coli</i> (EAEC)	-	-
Enteropathogenic <i>E.coli</i> (EPEC)	-	-
Enterotoxigenic <i>E.coli</i> (ETEC)	01	
Enteroinvasive <i>E.coli</i> (EIEC)	-	-

Figure.1 Multiplex PCR of Reference Strains and Clinical Samples



Lane 1: DNA molecular size marker (100bp ladder), Lane 2: Typical EPEC (*eaeA* amplicon size 229 and *bfpA* amplicon size 450), Lane 3: ETEC (*elt* amplicon size 322 and *Stla* amplicon size 170, Lane 4: EAEC (*CVD432* amplicon size 630bp), Lane 5: EHEC (*hlyA* amplicon size 534bp), Lane 6: EIEC (*ial* amplicon size 320), Lane 7 and 8: clinical isolates of EAEC (*CVD432*) and atypical EPEC (*eae*)

The Multiplex PCR assays were also used for detecting DEC directly from the stool samples which were positive for DEC. DNA was extracted from stool samples using QIAamp DNA Stool Mini Kit and subjected to multiplex PCR for detecting the presence of DEC. The multiplex PCR assays were found to be effective for direct detection of DEC in stool samples. The assays correctly identified 100% of the DEC strains directly in stool samples.

PCR is a highly sensitive and specific molecular biology technique for the detection of target DNA in various clinical specimens. Diarrheagenic *E.coli* is classified into five main categories according to the presence of different virulence genes (Moyo, 2007; Khairun, 2007; Brandal, 2007; Nguyen, 2005; Rajendran, 2010; Franzolin, 2005). In stool samples it can help to differentiate diarrheagenic *E.coli* strains from those of the normal flora (Moyo, 2007; Khairun, 2007; Brandal, 2007; Nguyen, 2005; Rajendran, 2010; Franzolin, 2005). DEC strains are usually characterized by phenotypic assays in most laboratories, but it is not possible to identify all the five pathotypes of DEC by these methods. DEC can be identified by molecular methods based on the presence of different chromosomal and/or plasmid encoded virulence genes that are absent in commensal *E.coli*. The genes that code for virulence factors of the different DEC have been extensively studied and characterized. Numerous PCR methods have been developed to identify the virulence genes of DEC. In this study we were able to detect DEC from pure cultures and spiked stool samples using control strains by the multiplex PCR assays. The limit of detection of DEC by the multiplex PCR assays was approximately 10^3 CFU/ml.

The prevalence and other epidemiological features of these pathogens as causative

agents of diarrhea vary from region to region. This variation is also seen between and within countries in the same geographical area. In this study four categories of DEC were detected in children with diarrhea. We found that ETEC was the most common DEC prevalent in children with diarrhea. Several recent studies have reported an increase in prevalence of atypical EPEC strains. Similarly in our study too most of the EPEC were of atypical type. Most of the earlier studies have used primers for ST gene only in the multiplex PCR assays. We subjected all our isolates to monoplex PCR assays using primers for both the subtypes. We detected only *Stla* gene in our isolates of ETEC by monoplex PCR assays, hence we included only detection of *Stla* gene in the multiplex PCR assay. In the present study we did not identify any EHEC EPEC EAEC strains. These results agree with the low prevalence of EHEC infection in developing countries. The low frequencies of EIEC strains are also in agreement with other studies performed in different parts of the world. There is not much data regarding previous studies using multiplex PCR assays for the detection of DEC from India.

The limitations associated with traditional diagnostic techniques can be overcome by PCR which is a sensitive, specific and rapid method for diagnosis. In this study, we were able to save time and effort involved in testing for virulence factors, reducing the number of gene detection assays by use of two sensitive and specific multiplex PCR assays. *E. coli* is considered as part of intestinal flora and no attempt is usually made for characterizing these strains further in routine diagnostic microbiology laboratory setting in a developing country. Accurate identification of DEC is important in understanding the disease spectrum and burden, tracing the sources of infection and routes of transmission. The rapid detection

of DEC has important treatment implications. Treatment of diarrhea does not usually depend on the etiological diagnosis, but such diagnosis bears an implication on overall management of patients. A clinical microbiology laboratory setting in a developing country could use these Multiplex PCR assays as a practical and rapid diagnostic tool for identification of DEC.

In conclusion, the multiplex PCR assays were able to correctly determine the presence of corresponding DEC virulence genes in all the reference strains of DEC. They can save considerable time and effort involved in testing for various virulence factors of DEC. Although singly they cannot be used for the detection of all strains of DEC, multiplex PCR assays could be used for detection of DEC in routine diagnostic laboratories. This study helped to understand that DEC does contribute to the burden of diarrhea in children in the developing world.

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How to cite this article:

Suganya, D., Kanimozhi, K., Panneerselvam, A. and Dhanapaul, K. 2016. Molecular Characterization of Diarrheagenic *Escherichia coli* from Tiruchirappalli District, Tamilnadu, India. *Int.J.Curr.Microbiol.App.Sci.* 5(3): 478-484.
doi: <http://dx.doi.org/10.20546/ijcmas.2016.503.056>