Original Article

Usefulness of a Multiplex PCR for Detection of Diarrheagenic *Escherichia coli* in a Diagnostic Microbiology Laboratory Setting

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Abstract

A multiplex PCR assay was evaluated for diagnosis of diarrheagenic *Escherichia coli* in stool samples of patients with diarrhoea submitted to a diagnostic microbiology laboratory. Two procedures of DNA template preparation-proteinase K buffer method and the boiling method were evaluated to examine isolates of *E. coli* from 150 selected diarrhoeal cases. By proteinase K buffer method, 119 strains (79.3%) of *E. coli* were characterized to various categories by their genes that included 55.5% enteroaggregative *E. coli* (EAEC), 18.5% enterotoxigenic *E. coli* (ETEC), 1.7% enteropathogenic *E. coli* (EPEC), and 0.8% Shiga toxin-producing *E. coli* (STEC). Although boiling method was less time consuming (<24 hrs) and less costly (<8.0 US $/ per test) but was less efficient in typing *E. coli* compared to proteinase K method (41.3% vs. 79.3% ; p<0.001). The sensitivity and specificity of boiling method compared to proteinase K method was 48.7% and 87.1% while the positive and negative predictive value was 93.5% and 30.7%, respectively. The majority of pathogenic *E. coli* were detected in children (78.0%) under five years age with 53.3% under one year, and 68.7% of the children were male. Children under 5 years age were frequently infected with EAEC (71.6%) compared to ETEC (24.3%), EPEC (2.7%) and STEC (1.4%). The multiplex PCR assay could be effectively used as a rapid diagnostic tool for characterization of diarrheagenic *E. coli* using a single reaction tube in the clinical laboratory setting.

Key words: Diarrhoea, diagnosis, *Escherichia coli*, multiplex PCR

Introduction

Diarrhoeagenic *Escherichia coli* is the most important etiologic agent of childhood diarrhea and represents a major public health problem in developing countries.[1] Identification of diarrheagenic *E. coli* strains requires differentiation from nonpathogenic members of *E. coli* that constitutes part of the normal enteric flora. Serogrouping of *E. coli* based on somatic O antigen used for differentiating diarrhoeagenic *E. coli* is costly, time-consuming and poorly correlates with presence of virulence factors.[2] Determination of virulence factors is thus essential for diagnosis of diarrheagenic *E. coli* strains. Polymerase chain reaction (PCR) has been successfully used to detect genes in many pathogenic bacterial species including diarrheagenic *E. coli*. A multiplex PCR for identification of enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC) and enteroinvassive *E. coli* (EIEC) has recently been developed.[3]

In the routine diagnostic microbiology laboratory setting in a developing country, *E. coli* is considered as part of intestinal flora and no attempt is usually made for characterizing these strains further. Accurate identification of diarrhoeagenic *E. coli* in a diagnostic laboratory setting is important in

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understanding the disease spectrum, tracing the sources of infection and routes of transmission and understanding the burden of the disease. Such identification would also assist the clinician to dispense appropriate management. However, for the diagnosis to be of pragmatic use to the patient, it is important that a diagnosis is provided as early as possible, preferably within 24 to 48 hours. Although, treatment of diarrhoea does not usually depend on the etiological diagnosis, but such diagnosis bears an implication on overall management of patients. The purpose of this study was to evaluate and adopt multiplex PCR in routine diagnostic microbiology setting for characterization of EAEC, ETEC, EPEC, STEC and EIEC in patients with diarrhoea, who submitted their stools or rectal swabs for routine cultures.

Methods

The study was conducted in the Molecular and Serodiagnostic unit of the Clinical Laboratory Services of Laboratory Sciences Division of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). The cases were included from inpatients admitted to Dhaka hospital of the ICDDR, B and the referral cases from other hospitals in Dhaka city between February and June, 2004. The clinical isolates of *E. coli* were obtained from routine cultures of diarrhoeal stools on MacConkey agar plates where *E. coli* grew as the sole bacterial colony and no Salmonella, Shigella or Vibrio species were isolated. The patients' demographic information on age and gender were available from laboratory records.

Briefly, stool or rectal swabs were cultured on MacConkey agar (MCA), Salmonella-Shigella agar (SSA) and Tellurite Taurocholate Gelatin agar (TTGA) plates and incubated aerobically at 37°C for 18 hours. The plates were screened for Salmonella, Shigella and Vibrio species. In absence of these pathogens, plates with sole growth of lactose-fermenting colonies on MCA were selected for identification of *E. coli* and subsequent characterization into their pathogenic types. Isolated single colony and a sweep of three colonies were propagated onto two fresh MCA plates and incubated at 37°C for 18 hours. Subsequently, a sweep of three colonies were inoculated in Luria-Bartoni (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and incubated overnight at 37°C with shaking.

Chromosomal DNA of *E. coli* was extracted from the single and sweep of three colonies grown on MCA plates using boiling method. Briefly, one loopful of *E. coli* from agar plates was suspended in 100 µl of sterile deionized water in an eppendorf tube and a bacterial suspension was made by vigorous shaking. The bacterial suspension was boiled for 10 minutes and chilled on ice followed by centrifugation at 10,000 x g for 1 min. The supernatant (75 µl) was used as DNA template for PCR.

Chromosomal DNA was also extracted from the sweep of three colonies on broth culture as described previously.[3] Briefly, thirty-six microliters (µl) of broth culture was added to 4 µl of 10X Tris-EDTA buffer and 60 µl of 2X Proteinase K buffer. After incubation for 90 min at 56°C in water bath, the sample was centrifuged at 10,000 x g for 1 min, and the supernatant (75 µl) was used as DNA template for PCR.

Multiplex PCR for categorization of *E. coli* into EAEC, ETEC, EPEC, STEC and EIEC was done using published primers for identification of *aggR*, CVD432 and *aspU* genes for EAEC, *elt* or *est* gene for ETEC, *eae* gene for EPEC, *eae* or *stx* genes for STEC and *ipaH* gene for EIEC.[3] The specificity of each primer set has been confirmed by monoplex PCR and then multiplex PCR was carried out using the reference strains with several PCR cycling protocol. Briefly, the optimized PCR protocol was carried out with a 50 µl mixture containing 10X PCR buffer, 50 mM MgCl$_2$, 2.5 mM dNTP 1.0 U of *Taq* DNA polymerase (Takara Bio Inc., Japan), 10 pmol concentrations of each primer set and 5 µl for DNA template. The PCR was carried out on Applied Biosystem 9700 thermal cycler using a program of initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min; and final extension at 72°C for 10 min. The PCR products were then separated by electrophoresis on a 2.0% agarose gel (AmpliSize; Bio-Rad Laboratories) stained with ethidium bromide and visualized by UV transillumination.

Results

During the study period, 7167 stool specimens were cultured. Of 150 specimens that yielded solely *E. coli* strains were

characterized by multiplex PCR. The proteinase K method for DNA extraction using sweep colonies characterized *E. coli* from 119 (79.3%) specimens, of which 66 (55.5%) were EAEC, 22 (18.5%) were ETEC, 2 (1.7%) were EPEC and 1 (0.8%) was STEC. Mixed infections were present in 28 (23.5%) cases. (Table I)

Table I. Types of *Escherichia coli* detected by multiplex PCR using boiling and proteinase K buffer for DNA extraction (n = 150)

Enteropathogenic *E. coli*	DNA extracted by Boiling n (%)	DNA extracted by proteinase K n (%)
EAEC	32 (51.61)	66 (55.46)
ETEC	21 (33.87)	22 (18.48)
EPEC	0	2 (1.68)
STEC	0	1 (0.84)
EIEC	0	0
MIXED	9 (14.52)	28 (23.53)
TOTAL	62 (41.33)	119 (79.33)

The boiling method using single and sweep colonies together identified various types of *E. coli* from 62 (41.3%) specimens, of which 32 (51.6%) were EAEC, 21 (33.9%) were ETEC and mixed infection was observed in 9 (14.5%) cases. However, single colony independently identified *E. coli* in 3 (4.8%) specimens only (2 were EAEC and one was ETEC), while sweep colonies identified *E. coli* in 13 (21.0%) specimens, of which 3 were EAEC, 1 ETEC and 9 in mixed (any two or more) infection. Of 43 ETEC strains identified by both methods, only 39 were independently identified. Among 39 ETEC isolates, ST gene was detected in 20 (51.3%), LT gene in 10 (25.6%) and both ST and LT gene was present in 9 (23.1%) isolates.

The overall detection rate of various pathogenic *E. coli* was higher with proteinase K method than the total detection by boiling method (79.3% vs. 41.3%; p<0.001). The sensitivity and specificity of boiling method compared to proteinase K method was 48.7% and 87.1% and the positive and negative predictive value was 93.5% and 30.7%, respectively. (Table II)

The age of the patients varied from 3 days to 97 years (mean age 8 years). Majority of patients (78.0%) were children around five years age and 53.3% were under one year and 68.7% of these children were male. (Table III)

Table II. Sensitivity, Specificity, Positive and Negative Predictive Values of DNA extraction by Boiling compared to Proteinase K in typing the *E. coli* isolates

		Proteinase K method		
		Pathogenic n (%)	Non-pathogenic n (%)	Total n (%)
Boiling method	Pathogenic	58 (48.7%)	4 (12.9%)	62 (41.3 %)
	Non-pathogenic	61 (51.3%)	27 (87.1%)	88 (58.7%)
	Total	119 (79.3%)	31 (20.7%)	150 (100%)

Table III. Distribution of patients by age and gender whose stool culture yielded *E. coli* as the sole isolate (n = 150)

Age group (years)	Male n (%)	Female n (%)	Total n (%)
< 1	55 (68.75)	25 (31.25)	80 (53.33)
1 - 5	23 (62.16)	14 (37.84)	37 (24.67)
6 - 15	4 (66.67)	2 (33.33)	6 (4.00)
16 - 25	4 (57.14)	3 (42.86)	7 (4.67)
26 - 45	6 (60.00)	4 (40.00)	10 (6.67)
> 45	4 (40.00)	6 (60.00)	10 (6.67)
Total	96 (64.00)	54 (36.00)	150 (100.00)

Of 74 children exactly under 5 years age, majority were infected with EAEC (71.6%), followed by ETEC (24.3%), EPEC (2.7%) and STEC (1.4%).

Discussion

Escherichia coli cause diarrhoea in humans through diverse mechanisms.[1] However, the pathogenic and non-pathogenic *E. coli* cannot be differentiated by the conventional diagnostic methods due to the lack of distinct phenotypic differences. It is the common practice in most of the clinical microbiology laboratory not to report *E. coli* as diarrhoeagenic pathogen as it is construed as a part of the normal intestinal flora. Characterization of *E. coli* is thus important, because a considerable number of pathogenic *E. coli* remains to be categorized by conventional diagnostic technique during the course of infection. Over the years, the diagnostic techniques have been improved and it has been shown that multiplex PCR assay could identify EAEC, ETEC, EPEC, STEC and EIEC, strains because of their well-defined virulence markers.[3]

In this study, about 80% of *E. coli* strains were identified as

belonging to one of the various types of diarrheagenic *E. coli*. Considering them as part of the normal flora and not accurately recognizing them as the cause of diarrhoea gives an inappropriate report. Of these *E. coli*, EAEC strains were the most common among the diarrheagenic patients especially among the children under 5 years age. The EAEC has been implicated as the etiological agent of diarrhea not only in developing countries but also as a cause of gastroenteritis outbreaks in some industrialized countries.[4] It has been reported as the cause of a massive outbreak of gastrointestinal illness in school children in Japan, as the cause of persistent diarrhea in children in Brazil, and in other developing countries.[5-8]

The second most common type of diarrheagenic *E. coli* was the ETEC. The ST genes of ETEC were found in majority of patients compared to the LT genes (51.3% vs. 25.6%; p <0.02). The ETEC has been attributed as the common cause of infections among the tourists visiting Asia, Africa and South America;[9] and also as a common diarrhoeal pathogen in children in many developing countries of Asia, Africa and South America.[10,11] One study in Bangladesh has reported ETEC infection as a cause of epidemic diarrhoea with a prevalence of 18.0%,[11] which correlated well with our findings (18.5%). There is also report of co-infection with EAEC and ETEC that accounted for 57.0% cases of the mixed infection[12] and also correlated with our study (60.7%).

The DNA extraction by proteinase K buffer using sweep colonies significantly yielded better result and seems to be more suitable for the multiplex PCR assay in characterizing the *E. coli* isolates compared to the boiling method. The sensitivity and the negative predictive value of boiling method are very low when compared to the proteinase K buffer method. There is significant difference between the use of single colony and sweep colonies (4.8% vs.21.0%; p < 0.01) for characterization of *E. coli* and may be a possibility that a single colony often does not capture the pathogenic strain. Therefore, the uses of sweep colonies of *E. coli* with proteinase K buffer remains a better option for determination of various types of diarrhoeagenic *E. coli*.

The overall time required to complete the characterization process and reporting to the physician is approximately 24 hrs and the approximate cost per assay is US$ 8.04, when such an assay is accomplished in a batch of at least five isolates of *E. coli*. A clinical microbiology laboratory setting, however, could undertake the characterization process of *E. coli*, if it has had some affordable equipment such as the DNA workstation, thermal cycler, gel electrophoresis with documentation system in addition to the required primers set of various pathogenic *E. coli* and other chemicals and reagents. This categorization of *E. coli* will facilitate to recognize the diarrhoeal etiology and assist physicians in deciding appropriate strategy for the management of diarrhoea in children less than five years, when no other pathogen is isolated or identified using conventional culture technique.

Infection with various types of diarrheagenic *E. coli* is common among the children and the majority of patients in our study were under five years age. Children when presented with diarrhoea and from whom no other etiologic agent is isolated, the sole growth of *E. coli* in the primary culture plates should be characterized for their diarrhoeagenic type. A clinical microbiology laboratory setting could use the Multiplex PCR assay as a practical and rapid diagnostic tool for the routine characterization of human diarrheagenic *E. coli,* if the cost is affordable. Further evaluation, however, using a broader sample size both from the hospital and community settings will facilitate to analyze the cost-effectiveness while considering its wider application in clinical settings.

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