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ORIGINAL ARTICLE

IDENTIFICATION OF ENTEROTOXIGENIC ESCHERICHIA COLI (ETEC) AND THEIR GENES: EST, HEAT-STABLE TOXIN (ST) AND ELT, HEAT-LABILE TOXIN (LT) USING MULTIPLEX PCR TECHNOLOGY IN ATERTIARY CARE HOSPITAL

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ABSTRACT

Despite the fact that diarrhaegenic Escherichia coli (DEC) has been identified as a major etiologic agent of childhood diarrhea which represent a major public health problem in developing countries, only a few studies have been performed in Bangladesh to identify these organisms. To detect Enterotoxigenic Escherichia coli (ETEC) in patients with acute diarrhea, a total of 450 stool specimens were tested by multiplex polymerase chain reaction (PCR). The multiplex PCR was designed for the detection of target genes of "elt" and "est" for enterotoxigenic E. coli (ETEC). Out of 450 stool specimens collected from patients with acute diarrhea, the ETEC was detected in 18% (81/450) cases. Both heat-stable toxin (ST) and heat-labile toxin (LT) genes of ETEC were detected in 64.20% (52/81) strains and only ST or LT as single gene was detected in 25.90% (21/81) or 9.90% (8/81) strains respectively. The multiplex PCR assay could be used as a rapid as well as efficient diagnostic tool for identification of ETEC in the clinical laboratory settings.

Key words: Diarrhoea, Enterotoxigenic Escherichia coli, Multiplex PCR.

INTRODUCTION

Acute infectious diarrhea (AID) is a major cause of morbidity and mortality worldwide. The AID remains a major public health challenge, especially in developing countries where it is a leading cause of death. Every year nearly 1.4 billion episodes of AID occur in children of less than 5 years of age in developing countries[1]. In addition diarrheal illness account for an estimated 12600 deaths each day in children of under 5 years of age in Asia, Africa and Latin America[2]. Kosek and associates (2003) reviewed studies from 1990 to 2000 and concluded that diarrhea accounts for 21% of all deaths under five years of age causing 2.5 million deaths per year in developing countries[1].

A diversity of recognized microorganisms such as bacteria, viruses and parasites may be involved in causing severe AID in children[3]. Numerous studies performed in different countries have reported diarrheagenic E. coli (DEC) as being the most frequent and important among bacterial pathogens associated with AID in developing countries. However, the frequencies of these pathogens vary with geographic region and depend on the socioeconomic/sanitary conditions[4].

In Bangladesh, the AID remains one of the most important health problems. One third of the total child death burden is due to diarrhea. Every year, a rural child suffers, on an average, from 4.6 episodes of diarrhea, out of which about 230,000 children die[5]. The diarrheagenic E. coli (DEC) has been reported to be responsible for 34% of diarrheal diseases in Bangladesh[6].

There are now at least six types of diarrheagenic strains of E. coli on the basis of distinct epidemiology and clinical feature, special virulence determinants and association

with certain serotypes. There are Enterotoxigenic E. coli (ETEC), Enteropathogenic E. coli (EPEC), Enterohemorrhagic E. coli (EHEC), Enteroaggregative E. coli (EAEC), Enteroinvasive E. coli (EIEC) and Diffusely Adhering E. coli (DAEC). Of these, EPEC, ETEC, EIEC, EHEC and EAEC are clearly associated with different types of enteritis, while the association of DAEC with diarrhea has not yet been clearly assessed, though it is potential pathogen. Further studies of DAEC are required to confirm its etiological role in diarrheal diseases.[7]

Due to lack of facilities, the ETEC cannot be detected in the routine diagnostic microbiology laboratory in developing countries, which is important in understanding the disease spectrum, tracing the sources of infection and the burden of the disease. Such identification would also assist the clinician to dispense appropriate management8. Identification of ETEC cannot be done only by culture and biochemical tests, since they are indistinguishable from the non pathogenic E. coli commonly found in human feces. Recently, various multiplex PCR methods have been developed for the detection of several specific genes of ETEC strains[9].

OBJECTIVES

The objective of the present study was to determine the prevalence of ETEC with their genes: est, heat-stable toxin (ST) and elt, heat-labile toxin (LT), in acute diarrheal patients using multiplex PCR as a diagnostic tool.

MATERIAL AND METHODS

This cross-sectional study was carried out during the period of January 2011 to December 2011 in the department of Microbiology, Mymensingh Medical College and included all patients with acute diarrhea irrespective of age and sex, admitted in Mymensingh Medical College Hospital. A total of 450 stool specimens were examined by standard laboratory methods for identification of E. coli. ETEC strains were detected by Multiplex PCR following standard methods.

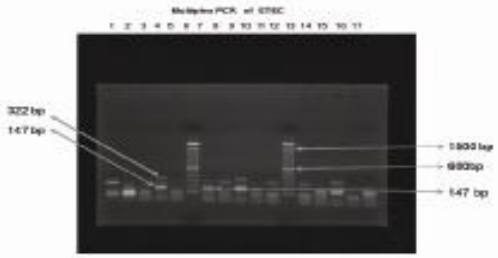
Briefly, all specimens were inoculated into Mac Conkey's agar (MCA) medium within 4 hours of collection and were incubated at 37…C for 24 hours aerobically. The isolated E. coli was identified on the basis of colony morphology, Gram staining, motility and biochemical tests.

DNA of E. coli was extracted from the sweep of few colonies grown on Mac Conkey's Agar plates. Briefly, one loopful of E. coli from agar plates was suspended in 100 l of sterile deionized water in an eppendorf tube and a bacterial suspension was made by using vortex. The bacterial suspension was boiled at 1000C for 10 minutes and centrifuged at 10,000 x g for 1 min. The supernatant was used as DNA template for PCR[8].

Table I: PCR primers were used for detecting Enterotoxigenic E. coli (ETEC), in the present study:

Designation -	Sequence (5 to 3) -	Target gene -size (bp) Amplicon	
AL65	TTA ATA GCA CCC GGT ACA AGC AGG	est	147
AL125	CCT GAC TCT TCA AAA GAG AAA ATT AC		
LTL	TCT CTA TGT GCA TAC GGA GC	elt	322
LTR	CCA TAC TGA TTG CCG CAA T		

ETEC was detected by Multiplex PCR using primers for identification of elt or est gene of ETEC (Table I). The PCR amplification was carried out with a 50 l reaction mixture containing buffer- 5 l, dNTP (0.2mM each)- 4 l, Primers (containing 30 p mol of each)- 3 l, Taq DNA polymerase (2.5 U)- 0.5 l, nuclease free water- 36.5 l and DNA tamplate- 1 l. The amplification was carried out on a thermal cycler (Eppendorf, Germany) using the following thermal and cycling conditions: initial denaturation at 95…C for 5 min, followed by 30 cycles, each containing denaturation at 95…C for 1 min, annealing at 56…C for 1 min, and extension at 72…C for 1 min; and final extension at 72…C for 10 min. The amplified products were then separated by horizontal electrophoresis on a 1.0% agarose gel, stained with ethidium bromide and visualized under UV Trans- illuminator. The amplicons were identified based on the size of the amplified product by comparing with 100 bp (base pair) DNA ladder marker[9].



Photograph of multiplex PCR showing est gene (147 bp) and elt gene (322 bp) of ETEC in lane 4, only est gene (147 bp) of ETEC in lane 6 and ladder marker (100bp) in lane 5 and 12.

RESULTS

In the present study, majority (67%) of the study population (302/450) belonged to <5 years of age, 21% (95/450) cases were in the age group <1 year and 46% (207/450) cases were in the age group 1-5 years. The rest 33% (148/450) cases were in the age group >5 years (Table II).

Table II: Age distribution of the study population:

Age group (years)	Number of patients	Percentage
<1	95	21.0
1-5	207	46.0
>5-15	86	19.6
>15-25	23	5.0
>25-35	21	4.6
>35-45	13	2.8
>45	5	1.0
Total	450	100.0

Of the 450 specimens examined, the ETEC was detected in 18% (81/450) cases. (Table: III). Both heat-stable toxin (ST) and heat-labile toxin (LT) genes of ETEC were detected in 64.20% (52/81) strains and as single gene, only ST and LT were detected in 25.90% (21/81) and 9.90% (8/81) strains respectively (Table: IV).

Table III: Detection rate of ETEC in the study population by multiplex PCR.

Number of samples tested samples	No. of ETEC positive	Percentage
450	81	18

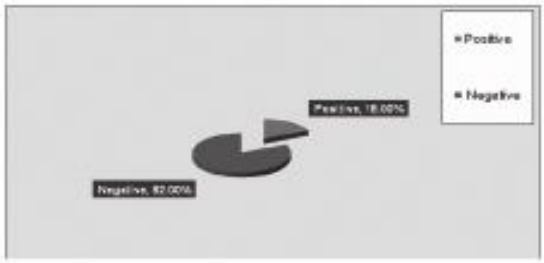


Figure: Detection rate of ETEC in the study population by multiplex PCR.

Table IV: Distribution of different genes (ST / LT/ Both) of ETEC (n= 81):

Type of gene (ST / LT/ Both) of Enterotoxigenic E. coli (ETEC)	Number of isolates	Percentage
Both (ST & LT) genes	52	64.2
ST gene	21	25.9
LT gene	08	9.9
Total	81	100

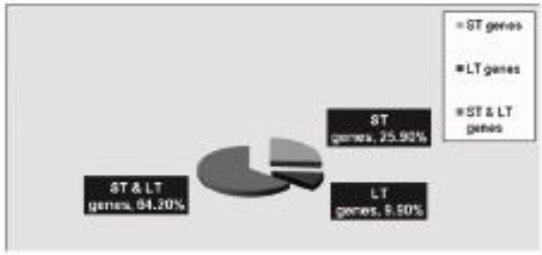


Figure: Distribution of different genes (ST / LT/ Both) of ETEC (n=81).

The highest detection of ETEC 44 (54.30%) was in the age group 1-5 years, followed by 21 (25.90%) in the age group >5 years and 16 (19.80%) in the age group <1 year (Table V).

Table V: Detection rate of ETEC in different age group (n=81).

Age group (years)	Number of ETEC positive cases.	Percentage
<1	16	19.80
1-5	44	54.30
>5-15	14	17.30
>15-25	05	6.20
>25-35	02	2.40
>35-45	0	0
>45	0	0
Total	81	100

DISCUSSION

Acute diarrhea is one of the most common illness and cause of death in young children in Bangladesh. Detection of ETEC, in the present study, is meaningful in that it provides information about the prevalence of ETEC in the study population.

In the present study, majority of the cases (67%) belonged to <5 years of age, in which 21% cases were in the age group <1 year and 46% cases were in the age group 1-5 years. The rest 33% cases were in the age group >5 years (Table II). Albert and associates (1999) as well as Stoll and associates (1982) reported that diarrhea was more common in children of <5 years of age in Bangladesh[10,11], which supports the present study findings. In a recent study from Vietnam by Nguyen and associates (2005), it was found that diarrhea was more frequent in children of less than 5 years of age[2], which correlates with findings of the present study.

In the study population, prevalence of ETEC was 18% (81/450) (Table III). In 2004, 2002 and 1999 it was reported that ETEC was responsible for 14.0%, 12.7% and 15.5% of diarrheal diseases in Bangladesh respectively[12,6,10], which were almost similar with the present study findings. Nessa and associates (2007) and Stoll and associates (1982) found ETEC was responsible for 19% and 20% of diarrheal diseases in Bangladesh respectively[8,11,13,14], which were slightly higher than the present study findings. In biological research some sorts of variation is not unexpected because it depends upon some factors like age, seasonality, methods of samples collection and tools used.

The ST gene of ETEC was found in majority of cases compared to LT gene. Out of 81 Enterotoxogenic E. coli, both (ST and LT) genes were detected in 64.20% (52/81) strains and only ST or LT as single gene was detected in 25.90% (21/81) or 9.90% (8/81) strains respectively (Table: IV). Significant association with diarrhea was seen with ETEC producing both ST and LT and ETEC producing only ST; ETEC producing only LT was not significantly associated with diarrhea. This trend agrees with the findings in other studies in Bangladesh by Qadri and associates (2000) and Albert and associates (1995)[12,15,17].

The highest detection of ETEC, 44 (54.30%) was in the age group 1-5 years, followed by 21 (25.90%) in the age group >5 years and 16 (19.90%) in the age group <1 year in the study population (Table V). Majority of ETEC (74%) belonged to <5 years of age. ICDDR, B (2002) as well as Qadri and associates (2000) reported that the most of the diarrheal patient, infected with ETEC were children <5 years of age in Bangladesh[6,12,16], which matches with the present study findings.

The prevalence of ETEC was studied previously in Bangladesh. However, these studies had several deficiencies, like some researchers relied on serotype and phenotype-based assay for ETEC, whose interpretation is prone to observer variation[18,19]. The present study overcomes all these deficiencies and shows that multiplex PCR is a suitable method for DEC detection by amplifying specific genes, where facilities are available.

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