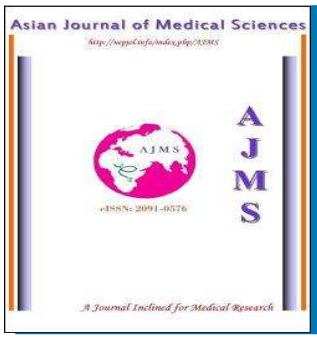

MOLECULAR DETECTION OF DIARRHEAGENIC *ESCHERICHIA COLI* FROM CHILDREN WITH ACUTE DIARRHEA IN TERTIARY CARE HOSPITALS OF DHAKA, BANGLADESH

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ABSTRACT

Objective: Multiplex PCR assay was used for diagnosis of diarrheagenic *Escherichia coli* (DEC) in stool samples of children (under 5 years) with acute diarrhea.

Methods: Samples were collected from January 2011 to December 2011, from Dhaka Medical College Hospital and Dhaka Shishu Hospital. Multiplex PCR with five specific primer pairs to detect enteropathogenic *E. coli* (*eae*, *bfp*), enterotoxigenic *E. coli* (*lt*, *st*) and enteroaggregative *E. coli* (*aat*) were used. However, enteroinvasive *E. coli*, enterohemorrhagic*E. coli* and diffusely adhererent*E. coli* were not sought.

Result: In total, 135 (67.5%) *E. coli* were isolated from 200 stool samples. The prevalence of DEC was 68 (34%). Among DEC, most frequently isolated pathotype was EPEC 40 (58.82%), followed by ETEC 24 (35.29%) and EAggEC 18 (26.47%). Among the EPEC, 5 (12.5%) were typical EPEC. Among the 68 DEC positive cases, 22 samples contained more than one pathogenic gene in various combinations. Among the combination of DEC, EPEC+ETEC combination was 6 (27.27%) followed by ETEC+EAggEC 4 (18.18%), EPEC+EAggEC and ETEC+EPEC+EAggEC were both in 3 (13.6%).

Conclusion: This study shows that DEC is a common cause of childhood diarrhea in Dhaka city of Bangladesh. By using multiplex PCR assay, DEC can be diagnosed in one PCR reaction that makes a conclusive diagnosis of diarrhea.

Keywords: *Escherichia coli*, Enteropathogenic *E. coli*, Enterotoxigenic *E. coli*, Enteroaggregative *E. coli*, Enteroinvasive *E. coli*, Enterohemorrhagic *E. coli*, Diffusely adhererent *E. coli,* Diarrhea, Polymerase chain reaction.

"By Multiplex PCR assay, DEC can be diagnosed in one PCR reaction that makes a conclusive diagnosis of diarrhea."

INTRODUCTION

Diarrhea continues to be one of the most common causes of morbidity and mortality among infant and children; especially in developing countries.[1] Diarrheal disease is the second leading cause of death and causes 1.3 million deaths every year in children less than five years.[2] In Bangladesh, one third of the total child death burden is due to diarrhea.[3] One in fifteen children die before their fifth birth day; among them diarrhea causes 7% of death.[4]

The causes of diarrhea include a wide range of viruses, bacteria and parasites.[5] Among the bacterial causes, Diarrheagenic *Escherichia coli* (DEC) is an important agent of childhood diarrhea and is a major public health problem in developing countries and is now being recognized as emerging entero-pathogen also in the developed countries.[6] It is responsible for 30%-40% of cases of acute diarrhea in children.[7] In Bangladesh, 41.33% to 46.78% cases of acute diarrhea in under 5 children are caused by DEC.[8,9] Based on their virulence factors, six recognized pathotypes for DEC are: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic*E. coli* (EPEC), Enteroaggregative *E. coli* (EAggEC), Enterohemorrhagic*E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC) and Diffusely adherent *E. coli* (DAEC).[10]

Identification of *E. coli* pathotypes in association with diarrhea is limited in many developing countries because conventional microbiological testing is unable to distinguish between normal flora and pathogenic strains of *E. coli*.[11] Accurate identification of DEC in a diagnostic laboratory setting is important in understanding the disease spectrum, tracing the sources of infection and routes of transmission.[8] Serotyping is the traditional method for detection of DEC. Although several serotypes are predominately found by the use of serotyping and correlated with specific categories of DEC, not all of the isolates belonging to those serotypes are truly pathogenic.[12,13] The antigen similarity may easily lead to false positive results in typing process.[14] The objective of the present study was to ascertain the association of various DEC with diarrhea in under 5 children in Bangladesh using multiplex PCR system.

MATERIALS AND METHODS

This was a cross sectional study conducted from January, 2011 to December, 2011. Stool samples were collected from 200 children (under 5 years) of acute diarrhea from outpatient department of Dhaka Medical College and Dhaka Shishu Hospital.

All stool samples were collected in a dry, clean, leak proof plastic container and brought to the microbiology laboratory of Dhaka Medical College within 2 to 4 hours. Cases were defined as children whose main complaint was acute diarrhea, characterized by the occurrence of three or more loose, liquid or watery stools with or without mucus and blood in a 24 hours period.

Ethics

The Ethical Review Committee (ERC) approved the protocol. Informed written consent was taken from parent or guardian before collection of sample.

Microbiological method

The samples were plated on MacConkey agar media incubated at 37^{0}C for 24 hours. Suspicious lactose fermenting colonies of *E. coli* were further subcultured onto Kligler-Iron agar, Motility-indole-Urea and Simmons citrate agar media. Further identification of the isolates was done by using standard biochemical methods.[15]

Molecular method

Multiplex PCR

After conformation, 3-5 colonies of *E. coli* were subcultured in triptic soy broth and incubated for 24 hrs at 37^{0}C. Bacterial pellets were formed by centrifuging the tryptic soy broth containing the growth of *E. coli* at 4000 rpm for 10 minutes. The pellets were resuspended with 300 µl sterile deionized water, boiled for 10 minutes and immediately kept on ice. It was again centrifuged at 14,000 rpm for 10 minutes. The supernatant was used as DNA template for PCR. The multiplex PCR was done by combining five primers listed in Table-1.

PCR was performed in a 25 µl reaction mixture containing 2 µl of extracted DNA, 12.5 µl mastermix-PCR buffer, dNTP, Taq polymerase (Promega corporation, USA), enzyme, $MgCl_2$ and loaded dye (Promega corporation, USA), 1 µl forward primer (0.5 µmol) and 1 µl reverse primer. Volume of the reaction mixture was adjusted by adding nuclease free water.

Amplification reactions were performed in a thermal cycler (Eppendorf AG, Mastercycler gradient, Hamburg,

Table 1.Sequence of Polymerase chain reaction primers, sizes of amplified DNA fragments

Gene	Sequence (5' - 3')	Product size (bp)	Reference
lt **(ETEC)**	5′TCTCTATGTGCATACGGAGC3′ 5′CCATACTGATTGCCGCAAT3′	322	**[23]**
st **(ETEC)**	5′GCTAAACCAGTAGAGGTCTTCAAAA3′ 5′CCCGGTACAGAGCAGGATTACAACA3′	147	**[23]**
bfp **(EPEC)**	5′TTCTTGGTGCTTGCGTGTCTTTT3′ 5′TTTTGTTTGTTGTATCTTTGTAA3′	367	**[23]**
eae **(EPEC)**	5′CCCGAAATTCGGCACAAGCATAAGC3′ 5′CCCGGATCCGTCTCGCCAGTATTCG3′	881	**[36]**
aat **(EAggEC)**	5′CTGGCGAAAGACTGTATCAT3′ 5′CAATGTATAGAAATCCGCTGTT3′	630	**[23]**

ETEC: Enterotoxigenic *E. coli*; EPEC: Enteropathogenic *E. coli*; EAggEC: Enteroaggregative *E. coli*.

in a thermal cycler (Eppendorf AG, Mastercycler gradient, Hamburg, Germany). Each PCR was carried out comprised of preheat at 94^0C for 10 minutes followed by 36 cycles of denaturation at 94^0C for 1 minute, annealing at 60^0C for 45 seconds, elongation at 72^0C for 2 minutes and final extension at 72^0C for 10 minutes.

Amplified products were analyzed onto horizontal gel electrophoresis in 1% agarose (Bethesda Research Laboratories) in 1X TBE buffer at room temperature at 100 volt for 30 to 35 minutes. A 100 bp DNA ladder was used as a molecular size marker in all gels. DNA bands were detected by staining with ethidium bromide and de-stained with distilled water. The amplified DNA bands were visualized by UV trans-illuminator and photographs were taken using digital camera (Gel Doc, Major science, Taiwan).

Statistics

All data were compiled and analyzed using Microsoft excels (2007). Statistical analysis was done by Chi-square test.

RESULTS

A total of 200 children with acute diarrhea were studied and *E. coli* were isolated by culture from 135 (67.5%) samples. Diarrheagenic *E. coli* were identified in 68 (50.37%) samples by multiplex PCR. The age of the patients varied from 6 months to 60 months as no samples were found from 0-6 months of age group. Among the study population the male-to-female ratio was 1.3:1. Here, x2 = 3.868, df=4, p=9.49, So, p>0.05, statistically not significant. Out of 68 DEC, 40 (58.82%) were males and 28 (41.18%) were females. The male to female ratio was 1.4:1. Here x2 = 6.72, df=8, p=15.51, p>0.05, statistically not significant. The incidence of different strains of *E. coli* according to sex groups showed that EPEC accounted for 40 (58.82%) of *E. coli* isolates with 24 (60%) males and 16 (40%) females. Out of 18 (26.47%) ETEC strains 11 (45.83%) were males and 13 (54.17%) were females. Among 18 (26.47%) of EAggEC, 10 (55.56%) were males and 8 (44.44%) were female. Table 2 demonstrates the incidence of each virulence genes among diarrheic patients. Age distribution of diarrheic patients is shown in Table 3.

Among isolated DEC, the most frequent pathotype was EPEC (58.82%), followed by ETEC (35.29%) and EAggEC (26.47%). Among 40 EPEC, atypical EPEC was detected in 35 samples (87.5%) and typical EPEC was detected in 5 samples (12.5%). Among 24 ETEC isolates, *st* gene was detected in 9 (37.5%), *lt* in 7 (29.17%) and both *lt* and *st* genes were present in 8 (33.33%) isolates. Out of 68 positive cases, 46 (67.65%) contained single gene and 22 (32.35%) samples contained more than one pathogenic genes. In the present study, distribution of different genes in various combinations is shown in Table 4.

DISCUSSION

DEC is an important and unrecognized cause of diarrhea in infancy, not only in developing countries but also in developed areas.[16] Diarrheagenic *E. coli* strains are being recognized as important pediatric enteropathogens worldwide.[17] Identification of *E. coli* pathotypes in association with diarrhea is limited in

Table 2. Incidence of each virulence gene among isolated *E. coli* (n=135).

Pathotype	Virulence gene	No. patients (%)
ETEC	*lt*	**7 (5.18)**
	st	**9 (6.67)**
	lt+ st	**8 (5.93)**
Atypical EPEC	*eae*	**20 (14.81)**
Atypical EPEC	*bfp*	**15 (11.11)**
Typical EPEC	*bfp+ eae*	**5 (3.70)**
EAggEC	*aat*	**18 (13.33)**

Table3. Distribution of age group with DEC.

Age (months)	No of Patients (%) (n=200)	No of DEC (%) (n=68)	ETEC (%) (n=24)	EPEC (%) (n=40)	EAggEC (%) (n=18)
6-12	98 (49)	36 (52.9)	8 (33.3)	19 (47.5)	**7 (38.9)**
13-24	47 (23.5)	10 (14.7)	7 (29.17)	15 (37.5)	**7 (38.9)**
25-36	22 (11)	8 (11.8)	5 (20.83)	3 (7.5)	**2 (11.1)**
37-48	15 (7.5)	7 (10.3)	2 (8.33)	0 (0.00)	**1 (5.6)**
49-60	18 (9)	7 (10.3)	2 (8.33)	3 (7.5)	**1 (5.6)**
Total	**200 (100)**	**68 (100)**	**24 (100)**	**40 (100)**	**18 (100)**

Table 4 Distribution of genes in various combinations among DEC positive cases (n=68)

Gene combinations	DEC	Samples N (%)
lt+st	ETEC	**2 (2.94)**
bfp+eae	EPEC	**4 (5.88)**
eae+aat	EPEC and EAggEC	**3 (4.41)**
lt+aat	ETEC and EAggEC	**3 (4.41)**
st+aat	ETEC and EAggEC	**1 (1.47)**
st+eae	ETEC and EPEC	**1 (1.47)**
st+bfp	ETEC and EPEC	**1 (1.47)**
lt+st+bfp	ETEC and EPEC	**1 (1.47)**
lt+st+eae	ETEC and EPEC	**2 (2.94)**
lt+eae+aat	ETEC, EPEC and EAggEC	**1 (1.47)**
lt+st+aat+bfp	ETEC, EPEC and EAggEC	**2 (2.94)**
lt+st+eae+bfp	ETEC and EPEC	**1 (1.47)**

In Figure A and Figure B the various bands of different types of DEC have been mentioned.

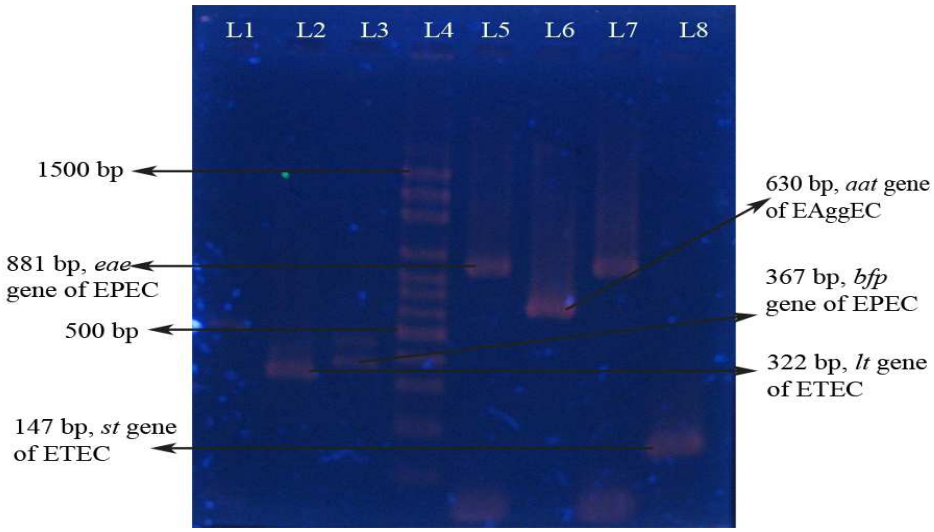

Figure A
Photograph shows bands of amplified DNA of DEC. *lt* gene of ETEC (laneL2), *bfp* gene of EPEC (lane L3), *eae* gene of EPEC (lane L5), *aat* gene of EAggEC (lane L6), *st*gene of ETEC (lane L8) and 100 bp DNA ladder (lane L4).

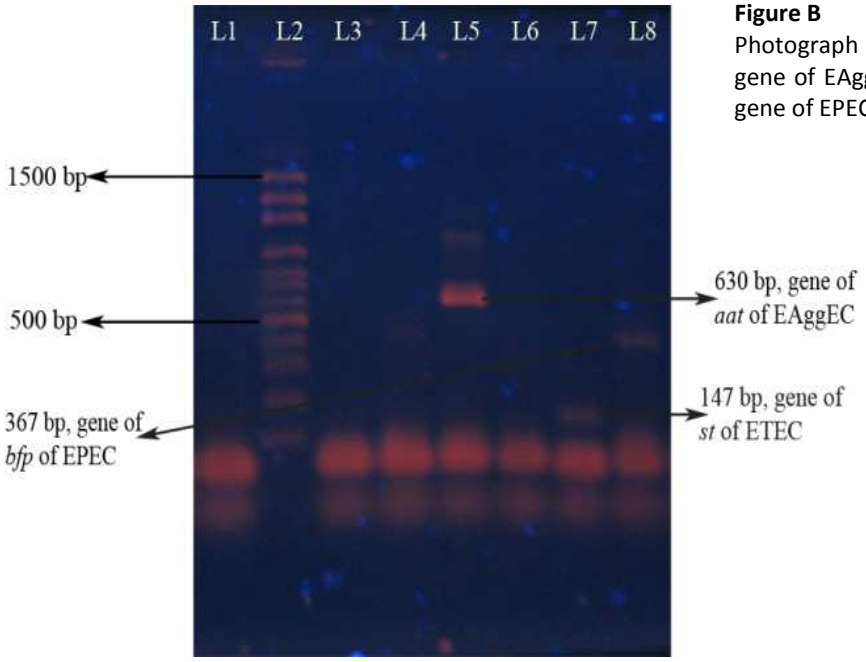

Figure B
Photograph shows bands of amplified DNA of DEC. *aat* gene of EAggEC (lanes L5), *st* gene of ETEC (lane L7), *bfp* gene of EPEC (lane L8), and 100 bp DNA ladder (lane L2).

many developing countries because conventional microbiological testing is unable to distinguish between normal flora and pathogenic strains of *E. coli*. [11]

In the present study, 68 (34%) isolates were identified as DEC by multiplex PCR. In previous studies in Bangladesh, 41.33% - 46.78% DEC were identified among diarrheic patients.[8,9] Similarly, in Iraq and Tanzania detection rate of DEC were 38% and 37.5%.[18, 19] However, the frequency of DEC varied in different countries, in Italy it was 6.3% [16], in India 19.44%,[20] in Mexico 16% [21] and in Taiwan 5.74%.[22] The prevalence and other epidemiological features of these pathogens as causative agents of diarrhea vary in the world from region to region and even between and within countries in the same geographical area.[23, 24]

In this study, 40 (58.82%) were identified as EPEC. In another study in Bangladesh, EPEC was also attributed to be a common (est) pathotype among DEC.[25] EPEC was also the most prevalent strain among DEC in Iraq, Riyadh, Iran, Brazil, Mexico and South Africa among diarrheal children.[19,26,27, 28] This high rate of identification of EPEC may be due to poor hygienic conditions, contamination of water supplies and overcrowding. Among the 40 EPEC strains, 5 (2.5%) was "typical EPEC" (contained both *eae* and *bfp*) gene, 35 (17.5%) were "atypical EPEC" (20 contained only *eae* gene and another 15 contained only *bfp* gene).[28] In the present study typical EPEC was less, a finding that is consistent with other reports. [16, 20]

In the present study, ETEC was the second 24 (35.29%) frequent type of DEC. In studies from Bangladesh and Iraq the frequency varied from 18% to 26% [8, 19, 29] whereas the frequency of ETEC was 51.6% in Tanzania.[17] The *st*genes of ETEC were found in 9 (4.5%) and *lt* gene was present in 7 (3.5%) and both *st* and *lt* genes were present in 8 (4%) of the study population. The predominance of *st* gene producing ETEC was also reported in various studies. [8, 19]

In the present study, 18 (26.47%) of EAggEC were detected among DEC. Similar trends were observed in studies in Brazil where 10% to 12% of EAggEC causing diarrhea was reported.[30, 31] EAggEC is emerging as an enteric pathogen of great concern and is responsible for acute and persistent diarrhea (≥ 14 days) and may cause malnutrition and growth defects in children. These strains have been associated with traveler's diarrhea in both developing and industrialized countries and have been isolated in immunocompromised patients.[27]

In the present study, among 68 DEC positive cases, 36 (52.94%) and 10 (14.71%) were from 6 to 12 and 13 to 24 months of age groups respectively and this difference was not statistically significant ($p>0.05$). A study in Bangladesh showed that 67% children below 24 months suffered from diarrhea caused by DEC.[8] A study from Vietnam also observed that DEC was significantly associated with diarrhea below two years.[14] The age-specific differences suggest that infants having immature immune systems may be exposed to contaminated formula milk, foods or environment or may have not been protected completely by breast feeding.[32]

In the present study, among 68 DEC positive cases, 40 (58.82%) were male and 28 (41.18%) were female and this difference was not statistically significant ($p>0.5$). The ratio of male and female was 1.4:1. The ratio of male and female of DEC positive cases in Tanzania were 1.6:1.[33] In a study in Nigeria the author also showed that sex had no effect on the distribution of diarrheagenic bacteria.[34]

In the present study, among 68 DEC positive cases, 22 (32.35%) contained more than one pathogenic genes of DEC in various combinations. Similarly, in Nicaragua, among co-infections, the combinations were EPEC+EAggEC in 15 (18.52%), ETEC+EAggEC in 11 (13.58%), ETEC+EPEC in 5 (6.17%) and ETEC+EPEC+EAggEC in 3 (3.7%) [35] and in Tanzania, EPEC+EAggEC combination was in 2 (22.22%) and ETEC+EAggEC combination was in 3 (33.33%) samples. [34]

This study shows that DEC is a common cause of childhood diarrhea in Dhaka city of Bangladesh. By using multiplex PCR assay, DEC can be diagnosed in one PCR reaction that makes a conclusive diagnosis of diarrhea. If in clinical laboratory setting, detection of DEC by using multiplex PCR in routine practice it would be a fruitful achievement to treat acute diarrheic children.

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Authors Contributions:

SR: Concept and Design of the study and manuscript writing.

SMS & KM: Co-operation during laboratory work and write up the article.

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