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Diarrhoeagenic *Escherichia Coli* Isolated from Children with Acute Diarrhoea by Culture and Multiplex PCR in Tertiary Care Hospitals of Dhaka, Bangladesh

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Abstract

Background: Diarrhoeal diseases are a major childhood health problem and an important cause of morbidity and mortality, especially in developing countries. **Objective:** This study aimed to detect Diarrhoeagenic *Escherichia coli* (DEC) by detecting virulence genes (lt, st, bfp, and aat) and to determine the antimicrobial susceptibility pattern of all identified DEC isolates in fecal samples. **Methodology:** This cross-sectional study was carried out in the Department of Microbiology at Dhaka Medical College Hospital, Dhaka, Bangladesh and Dhaka Shishu Hospital, Dhaka, Bangladesh from July, 2012 to June, 2013 for around one year. Identification of Diarrhoeagenic Escherichia coli (DEC) in fecal samples from children under 5 years old was done by culture and standard biochemical tests. Virulence factor genes for DEC were detected by Polymerase Chain Reaction (PCR). Susceptibility to antimicrobial agents of all identified DEC isolates was observed by disc diffusion method. **Results:** Out of total 200 stool samples, *E. coli* was isolated in 130(65.0%). The prevalence of DEC was 52(40.0%) and the most common was enterotoxigenic Escherichia coli (55.8%) followed by enteropathogenic Escherichia coli (34.6%) and enteroaggregative Escherichia coli (26.9%). Among 52 DEC, 12(23.1%) contained more than one pathogenic genes of DEC in various combinations. Colistin, imipenem and amikacin were found to be the most effective against isolated DEC. Most of the strains were resistant to ampicillin, erythromycin, tetracycline and nalidixic acid. **Conclusion:** Differentiation between the Diarrhoeagenic Escherichia coli pathotypes is of great importance since they are involved in acute diarrhoeal diseases and may require specific antimicrobial chemotherapy.

Keywords: Diarrhoeagenic; *Escherichia coli*; diarrhoea; antimicrobial resistance

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Introduction

Diarrhoeal diseases are one of the major public health problems in developing countries and around 1.5 million deaths occur annually due to diarrhoea[1]. It is consider as the second-highest cause of death among under five years children and it also causes 525,000 deaths in children every year[2]. Among the bacterial causes, Diarrhoeagenic *Escherichia coli* (DEC) is one of the most important agent of childhood diarrhoea in developing countries[3]. In Bangladesh, 41.33% cases of acute diarrhoea in under five children are caused by DEC[4].

The classification of Diarrhoeagenic *Escherichia coli* strains is based on their virulence properties and comprises six groups: enterotoxigenic Escherichia coli (ETEC), enteropathogenic Escherichia coli (EPEC), enteroinvasive *Escherichia coli* (EIEC), enterohaemorrhagic *Escherichia coli* (EHEC),

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enteroaggregative *E. c*oli (EAEC) and diffusely adherent *Escherichia coli* (DAEC)[5]. Cytolethal distending toxin-producing *E. coli* (CDT-EC) have also been described as diarrheagenic[6]. ETEC is the most frequently isolated enteropathogen annually affecting up to 400,000,000 children under 5 years of age living in developing countries[7]. Enteropathogenic Escherichia coli (EPEC) are one of the main causes of persistent diarrhoea[8]. EAggEC is an increasingly recognized enteric pathogen and is the cause of acute or persistent diarrhoea in children and adults in both developed and developing countries[9].

Resistance of antibiotics is very common in bacterial isolates all around the world[10]. So information on antimicrobial resistance patterns is important in choosing the most appropriate antibiotic therapy. *Escherichia coli* is one of the most important opportunistic pathogen that has shown an increasing antimicrobial resistance to most of antibiotics[9].

A large proportion of *Escherichia coli* are multidrug resistant (MDR) and they could be a source of spread of these pathogens to their contacts[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[4]. The objective of the present study was to ascertain the association of various DEC with diarrhoea in under 5 children in Bangladesh and also aimed to see the antimicrobial susceptibility pattern of the isolated organisms.

Methodology

Study Settings and Population: This cross-sectional study was conducted from July 2012 to June 2013 in the Department of Microbiology at Dhaka Medical College, Dhaka, Bangladesh. Children aged 6 months to 5 years with acute watery diarrhoea from Dhaka Medical College Hospital, Dhaka, Bangladesh and Dhaka Shishu Hospital, Dhaka, Bangladesh with or without mucus and blood were included in this study. Patients treated with antibiotics within last 7 days were excluded from this study.

Study Procedure: Stool samples were collected from 200 children (under 5 years) of acute diarrhoea. Samples inoculated on agar media and suspicious colonies of *Escherichia coli* were identified by their colony characteristics, Gram staining and standard biochemical tests[12]. Multiplex PCR for categorization of Escherichia coli was done using primers for detection of lt and st gene for ETEC, bfp gene for EPEC and aat gene for EAggEC[13]. Susceptibility to antimicrobial agents of *Escherichia coli* isolates were done by Kirby Bauer modified disk diffusion technique using Mueller Hinton agar plates. The antibiotics used were ampicillin, tetracycline, ciprofloxacin, cotrimoxazole, nalidixic acid, azithromycin, ceftriaxone, ceftazidime, cefixime, cefuroxime, chloramphenicol, amikacin, imipenem, amoxiclav and colistin[14].

Procedure of Culture: The samples were plated on MacConkey agar media, incubated at 37º C for 24 hours. Suspicious lactose fermenting colonies of *Escherichia coli* were further sub-cultured onto Kligler-Iron agar, Motility-indole-Urea and Simmons citrate agar media.

Procedure of Multiplex PCR: After conformation, 3-5 colonies of isolated bacteria were sub-cultured in triptic soy broth. Bacterial pellets were prepared by centrifuging the tryptic soy broth containing the growth of bacteria at 6000 g for 10 minutes. The pellets were resuspended with 300 µl of sterile deionized water, vortexed until mixed, then heated at 100°C for 10 minutes in a heat block and immediately placed on ice for 5 minutes and then centrifuged at 14,000 g at 4°C for 10 minutes. Supernatant was used as DNA template. The primers were,

lt- F: 5'-TCTCTATGTGCATACGGAGC-3' and R: 5'-CCATACTGATTGCCGCAAT-3' for lt gene of ETEC, st- F: 5'-GCTAAACCAGTAGAGGTCTTCAAAA-3' and R: 5'- CCCGGTACAGAGCAGGATTACAACA-3' for st gene of ETEC, bfp- F: 5'-TTCTTGGTGCTTGCGTGTCTTTT-3' and R: 5'-TTTTGTTTGTTGTATCTTTGTAA-3' for bfp gene of EPEC, aat- F: 5'-CTGGCGAAAGACTGTATCAT-3' and R: 5' CAATGTATAGAAATCCGCTGTT5'-3' for aat gene of EAggECH.

PCR was performed in a 25 µl reaction mixture containing 2 µl extracted DNA, 12.5 µl mastermix and loaded dye (Promega corporation, USA), 2 µl forward primer and 2 µl reverse primer and 6.5 µl nuclease free water. After a brief vortex, the tubes were centrifuged for a few seconds. Each PCR was carried out comprised of preheat at 94º C for 10 minutes followed by 36 cycles of denaturation at 94º C for 1 minute, annealing at 58º C for 45 seconds, elongation at 72º C for 2 minutes and final extension at 72º C for 10 minutes. Amplified products were analysed on electrophoresis on 1% agarose gel at 100 volt for 35 minutes. A 100 bp DNA ladder was used as a molecular size marker in all gels. Gel was stained with ethidium bromide. The amplified DNA bands were

visualized by UV trans-illuminator.

Statistical Analysis: Data were compiled and analysed using Microsoft Excel (2007); comparisons were performed by Z test.

Ethical Clearance: All procedures of the present study were carried out in accordance with the principles for human investigations and also with the ethical guidelines of the Institutional research ethics. Ethical clearance to carry out this study was obtained from The Ethical committee of Dhaka Medical College, Dhaka, Bangladesh. Participants in the study were informed about the procedure and purpose of the study and confidentiality of information provided. All the participants consented willingly to be a part of the study during the data collection periods.

Results

Out of 200 suspected children, age varied from 6 months to 60 months as no sample was found from 0-6 months of age group. Among the study population, the male to female ratio was 1.2:1 (Table-1).

Table 1: Age and sex distribution of study population (n=200)

Age Group	Male	Female	Total
6 to 12 Months	54(27%)	49(24.5%)	103(51.5%)
13 to 24 Months	22(11%)	10(5%)	32(16%)
25 to 36 Months	14(7%)	11(5.5%)	25(12.5%)
37 to 48 Months	10(5%)	12(6%)	22(11%)
49 to 60 Months	10(5%)	8(4%)	18(9%)
Total	**110(55.0%)**	**90(45.0%)**	**200(100.0%)**

Male : Female = 1.2:1

E. coli were isolated by culture from 130 (65%) samples. Diarrhoeagenic *E. coli* were identified in 52 (40%) samples by multiplex PCR (Figure I).

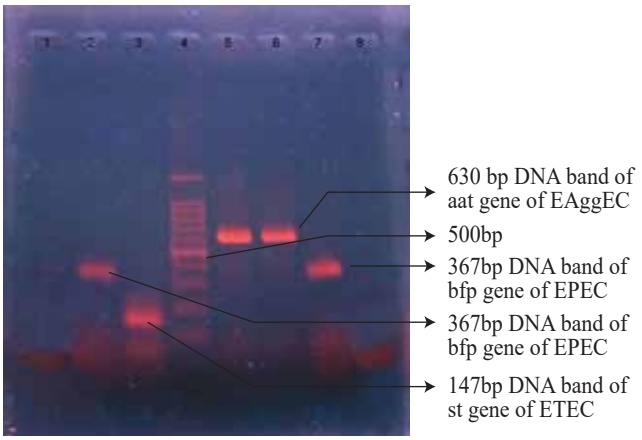

Figure I: Photograph showing bands of amplified DNA of DEC; lane 2: lt gene of ETEC, lane 3: st gene; lane 4: 100bp DNA ladder; lane 5,6: aat gene of EAggECH; lane 7: bfp gene of EPEC

Among isolated DEC, the most frequent pathotype was ETEC (55.77%), followed by EPEC (34.62%) and EAggEC (26.92%). Among 29 ETEC isolates, 13(44.83%) had only *st* gene, 11 (37.93%) had only *lt* gene and 5(17.24%) had both the genes together. Among 52 DEC positive cases, 40(76.92%) contained single gene and 12(23.07%) contained more than one pathogenic genes of DEC in various combinations (Table-2).

Table 2: Distribution of different genes in combination among the isolated DEC (n=52)

Gene Combinations	DEC	Samples
lt + st	ETEC	4 (7.69%)
lt + aat	ETEC and EAggEC	1 (1.92%)
st + aat	ETEC and EAggEC	1 (1.92%)
st + bfp	ETEC and EPEC	1 (1.92%)
lt + bfp	ETEC and EPEC	2 (3.84%)
bfp + aat	EPEC and EAggEC	1 (1.92%)
lt + st + bfp	ETEC and EPEC	1 (1.92%)
lt + bfp + aat	ETEC, EPEC and EAggEC	1 (1.92%)

Among the 52 isolated DEC, 50 (96.15%) were sensitive to colistin followed by 49 (94.23%) to imipenem, 45 (86.54%) to amikacin, 32 (61.54%) to amoxiclav, 40 (76.92%) to ceftazidime and co-trimoxazole. Resistance rate to ciprofloxacin, tetracycline, nalidixic acid, ampicillin, erythromycin were 29(55.77%), 43(82.69%), 32(61.54%), 47(90.38%) and 42(80.77%) respectively (Table-3).

Table 2: Antimicrobial Susceptibility Patterns of Isolated DEC

Name of Antibiotics	Sensitive	Resistant
Colistin	50(96.15%)	2 (3.85%)
Imipenem	49(94.23%)	3(5.77%)
Amikacin	45(86.54%)	7(13.46%)
Amoxiclav	32(61.54%)	20(38.46%)
Ceftazidime	40(76.92%)	12(23.08%)
Ceftriaxone	22(42.31%)	30(57.69%)
Cefixime	20(38.46%)	32(61.54%)
Cefuroxime	24(46.15%)	28(53.85%)
Ciprofloxacin	23(44.23%)	29(55.77%)
Azithromycin	27(51.92%)	25(48.08%)
Tetracycline	9(17.31%)	43(82.69%)
Nalidixic acid	20(38.46%)	32(61.54%)
Chloramphenicol	15(28.85%)	37(71.15%)
Ampicillin	5(9.61%)	47(90.38%)
Co-trimoxazole	40(76.92%)	12(23.08%)

Discussion

DEC is an important and unrecognized cause of diarrhoea in infancy, not only in developing countries but also in developed areas. Identification of *E. coli*

pathotypes in association with diarrhoea is limited in many developing countries because conventional microbiological testing is unable to distinguish between normal flora and pathogenic strains of *E. coli*[7].

Among DEC, ETEC was found as the most frequent *E. coli* pathotype which correspond to 55.77%. As contaminated food and water sources both contribute to ETEC infections, inadequate safe water, poor food hygiene and sanitation of our country might be the reason for high prevalence of ETEC diarrhoea. ETEC was also reported as the most frequent bacterial cause of diarrhoea in other studies[15-16]. On the contrary, some reports from Bangladesh showed much lower rates of ETEC among DEC[17]. In this study, strains carrying only the st gene were more prevalent (44.83%) than those carrying genes for lt (37.93%) or both the genes together (17.24%) which are in accordance with other reports[4,18].

In the present study, EPEC (34.62%) was the second most frequent type of DEC which correlates with another study[19]. On the contrary, some studies have reported much higher rate of 58.82% to 63.1% EPEC as the most frequent type of DEC associated with diarrhea[16,20]. Variation in the prevalence of ETEC toxin types or different categories of *E. coli* may occur from year to year and among different geographical areas.

Low rates of resistance to newer drugs like colistin and imipenem were found in this study which may be also due to the fact that injectable forms of these drugs are not usually used without physician prescription. Until now older antimicrobials like ampicillin, tetracycline, erythromycin are frequently used to treat diarrhoea in children though the drugs are found highly resistant. The present study is in accordance with several other studies that reported increasing resistance of DEC to these commonly used antibiotics[21-22].

Diarrhoeal diseases are common in Bangladesh and as the antibiotics are easily available and sold over the counter, so anyone can get it without physician prescription. So indiscriminate use of these antimicrobials, self-medication, in-appropriate and incomplete dose schedule, overprescribing and improper selection of antibiotics in diarrhoea patients might have helped in developing resistance to these commonly used antibiotics.

Conclusion

This study determined the significance and the association of DEC as important aetiological agents of bacterial diarrhoea in children younger than 5 years. The patterns of high rates of resistance to commonly used antimicrobials for treatment of diarrhoea imply an urgency to control the extent of indiscriminate use of various antimicrobial agents, regular monitoring of antimicrobial resistance pattern and rational use of various antimicrobial agents. The detection rate of multiplex PCR assay is rapid, easy to perform and the overall excellent specificity allows it for characterization of DEC. Development of multiplex PCR system at tertiary level hospitals is needed to reduce the cost and workload to make a conclusive diagnosis of diarrhoea.

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Conflict of Interest
The authors declared no conflict of interest.

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Authors' contributions
Rabin N and Shamsuzzaman SM conceived and designed the study, analyzed the data, interpreted the results, and wrote up the draft manuscript. Yusuf MA, Shams F, Tarana MN contributed to the analysis of the data, interpretation of the results and critically reviewing the manuscript. Wasimuddin SM involved in the manuscript review and editing. All authors read and approved the final manuscript.

Data Availability
Any inquiries regarding supporting data availability of this study should be directed to the corresponding author and are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate
Ethical approval for the study was obtained from the Institutional Review Board. As this was a prospective study the written informed consent was obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations.

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