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CLONAL RELATIONSHIP ASSESMENT THROUGH PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF DIFFERENT VIROTYPES OF *ESCHERICHIA COLI* PRESENT IN DIARRHEAL PATIENTS AND NON-DIARRHEAL CONTROL

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ABSTRACT
Escherichia coli is one of the most prevalent causes of diarrhea throughout the world including Bangladesh. This study has been undertaken to investigate the prevalence of different virotypes of *E. coli* among diarrheal patients and non-diarrheal controls and to assess clonal relationship phenotypically and genotypically. A total of 166 stool samples were collected from both diarrheal patients (n=71) and non-diarrheal controls (n=95). From these, 15 virulent *E. coli* (15.8%) were isolated from non-diarrheal controls and 18 virulent *E. coli* (25.4%) were isolated from diarrheal patients following standard microbiological and biochemical methods. These isolates were screened for the prevalence of different virotypes of *E. coli* by PCR method. Based on PCR results , most prevalent *E. coli* virotype among the diarrheal samples were EAEC (11.27%), followed by ETEC (8.45%) and EPEC (5.63%) whereas in non-diarrheal controls the most prevalent was EPEC (7.46%) followed by ETEC (4.5%) and EAEC (4.5%). None of the samples were positive for *ial*, which confirmed the absence of EIEC in this study. In plasmid profile analysis different plasmid patterns were found in the EPEC, ETEC and EAEC strains. Middle ranged plasmids (60 MDa to 90 MDa) were found to be present in 78.78% strains. All typical EPEC strains harbored the 60 MDa to 90 MDa plasmid known as EPEC adherence factor (EAF) plasmid. Antibiotic susceptibility test showed that most of the *E. coli* strains (66.7%) isolated from diarrheal patients was resistant to Amoxicillin, Mecillinam and Trimethoprim-sulfamethoxazole; the same result was found with strains from non-diarrheal controls. Of all the 33 strains, 3 were resistant to Ciprofloxacin (10%). All EAEC strains from both diarrheal patients and non-diarrheal controls showed heterogenous banding pattern in PFGE analysis suggesting diverse clonal relationship among them whereas among the EPEC strains, interestingly 3 strains (1 from diarrheal patient and 2 from non-diarrheal controls) were clonally identical. This study will help to identify how non-pathogenic EPEC strains become pathogenic.

KEYWORDS: EAEC, EAEC, EPEC, EIEC, PCR, Plasmid, Clonal relationship.

INTRODUCTION

Diarrheal diseases remain a leading cause of morbidity and mortality in the world and it is responsible for malnutrition in children in the under five age group particularly in developing country (Adachi *et al.*, 2001; Ogata *et al.*, 2002; Robins-Browne & Hartland, 2002). It has been estimated that 2 billion to 4 billion episodes of infectious diarrhea and 3 million to 5 million deaths from diarrhea occur annually in developing countries (Sanchez *et al*., 2005). *Escherichia coli* is a versatile organism that can cause secretory and bloody diarrhea as well persistent diarrhea (Mark *et al.*, 2016). The pathogenesis of the different serotype of *E. coli* is self explanatory in producing different type of diarrhea. The diarrhea caused by *E. coli* is a worldwide phenomenon from Middle East to Latin America and from Europe and Asia to Africa and affects both the developed and developing country(Qadri *et a*l., 2005) Humans and most worm blooded animals carry *E. coli* as a part of the normal intestinal flora. This facultative anaerobic is a non spore-forming gram negative bacillus, fimbriated, flagellated and capsulated. A group of these organisms have developed the ability to cause both intestinal and extra intestinal disease.(Robins-Bromne *et al*., 2002, Nataro *et al*., 1998) Strains associated with the presence of diarrhea hve been grouped according to shared pathogenic characteristics.(Kaper *et al*., 2004) To date, the five major groups are 1) Enterotoxignic (ETEC) that causes infantile and traveler's diarrhea; infant diarrhea; 2) Enteropathogenic (EPEC) that causes infant diarrhea; 3) Enteroinvasive (EIEC) that cause dysentry; 4) Enteroaggregative (EAEC) that are associated with bloody and persistent diarrhea and 5) enteropathogenic (EHEC) that cause hemorrhagic colitis and hemolytic urmic syndrome.(Levine, 1987) These different clinical manifestations are related with the presence of virulence genes present in plasmids and in defined groups of genes, called pathogenicity islands, present in the chromosomes of these bacteria. The proteins coded by these genes allow the bacteria to interact with the cells in the intestinal mucosa and disrupt normal functions with the consequent expression of clinical symptoms. (Gilligan *et al.*, 1999) These Bacteria are present in uncooked foods (Belongia *et al*., 1991) and in intimate environment (Brewster *et al.*, 1994) and has been identified as an unrecognized cause of childhood diarrhea in both developed and developing countries. The global estimates of diarrhea due to ETEC and EPEC is the highest among the under five children. In the year 2000 the global share of EPEC and ETEC

diarrhea in the community were 22.9% while it was 17.8% in the outpatient department and 25.1% in the inpatient department of the hospitals (Lanata *et al*., 2002). This is first time where PFGE clonal relationship pattern of non-diarrheal control has been compared with diarrheal samples. This study may be helpful to identify how non-pathogenic EPEC strain becomes pathogenic although two EPEC strains of non-diarrheal control have same phenotypic and genotypic characters of one diarrheal EPEC strain. These findings can be merged with the findings of pathogenicity pattern of Kallonen, T and Boinett, C. J., 2016; Croxen, M.A. and Finlay, B.B., 2010 for better understanding of EPEC pathogenicity.

MATERIALS & METHODS

This case control study was performed with stool samples collected from both non-diarrheal controls and diarrheal patients. A total of 166 stool samples were examined of which 91 were collected from non-diarrheal controls (CMS) and 72 were collected from diarrheal patients (CDS). From stool samples *E. coli* were initially identified using stasndard microbiological and biochemical methods (WHO, 1999). A loop full colony of confirmed *E. coli* spp. isolates were grown in Trypticase Soy Agar (TSA) overnight at 37oC and were stored in Trypticase Soy Broth (TSB) with 0.3% yeast extract and stored at -70ºC after addition of 15% glycerol for further use. *Escherichia coli* strains PDK-9, V-517 and R1 were used as plasmid molecular weight standard. *Salmonella braenderup* was used as PFGE molecular weight standard. All these strains which were used as standard were collected from the Enteric Microbiology Lab, Laboratory Science Division, ICDDR, B, Dhaka.

Phenotypic Characterization

Bacterial susceptibility to antimicrobial agents by the disk diffusion method

Bacterial susceptibility to antimicrobial agents was determined by the disk diffusion method as recommended by the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards, 1999) with commercial antimicrobial discs (Oxoid, Basingstoke, UK). The antibiotic discs used in this study are above.

Molecular Characterization

Detection of Specific Genes by PCR Assay

Representative isolates were grown on Tripton Soy agar for overnight. A loop full colony of each isolate was suspended in 1ml distilled water into an eppendorf tube. Then vortex the mixture and boil for about 20min. After boiling, the tube is immediately ice cooled. Cool down for about 10min and then centrifuge at 3000rpm for 10min and finally take the supernatant and the template DNA is ready for multiplex PCR. The respective 3µl template DNA was suspended in 25 µl of reaction mixer containing 2.5 µl of 10X PCR buffer with MgCl2, 2 µl of 1.25 mM dNTP, .2 µl *lt* and *st* (ETEC) encoded primer (forward and reverse), .4µl of *bfp* (EPEC) and *aat* (EACE) encoded primers, 0.22µl of *eae* (EPEC) encoded primers, 0.22µl of *ial* (EIEC) encoded primers together with 1 unit of Taq DNA polymerase (5 U/µl). Volume of the reaction mixture was adjusted by adding filtered deionized water. The reaction mixer was overlaid with a drop of mineral oil in order to prevent condensation. PCR assays were performed in a DNA thermal cycler (model 480; Perkin-Elmer Cetus, Emeryville, USA) Each PCR test used the same basic set-up: 96ºC for 4 min followed by 34 cycles of 20 sec at 95ºC, 20 sec at $T_{Annealing}$ at 57ºC for 20 sec and $T_{Elongation}$ (min) at 72ºC for 1min with a final extension at 72ºC for 7 min. A reagent blank, which contained all components of the reaction mixture with the exception of the bacteria, was included in every PCR procedure. ATCC *E. coli* (25922) strain was used as negative control for all PCR. *E. coli* AD9769C2, *E. coli* K-100 was used as positive controls for *eae*, lt, st gene respectively. Amplification products were subjected to horizontal gel electrophoresis in 1% agarose gel in TBE (Tris-borate EDTA) buffer at room temperature at 100 volt (50 mA) for 1h.

Plasmid Profiling

An isolated colony of each isolate was inoculated into 1.5 ml of TSB broth with 0.3% yeast extract (YE) and incubated overnight at 37ºC on a water bath shaker. Cells were collected in a polypropylene microcentrifuge tube by centrifuging the broth culture in an Eppendorf centrifuge (Model No. 5415 C) at 14,000 rpm for 5 min Supernatant was removed and the pellet was suspended in 100µl of solution I (40 mM tris-NaOAc, 2 mM EDTA, pH 7.4) by vortexing. Then 200 µl of solution II (3% SDS, 50 mM tris, pH 12.6) was added and was mixed gently by rapid inversion of the tube and was incubated at 55ºC for 45 min in a water bath. After incubation, the tubes were taken out and an equal volume of solution III (300 µl) (phenol: Chloroform: Isoamylalcohol as 25:24:1) was added and mixed well by slowly inverting the tubes until a milky white suspension was formed. Then the tubes were centrifuged at room temperature for 8 min at 14,000 rpm. It formed three layers, the upper layer was the plasmid solution, middle layer consisted of cell debris together with other proteinatious fractions, and the lower layer was the phenol. Using a Pasteur pipette the plasmid solution was removed carefully and transferred into a new eppendorf tube. Plasmid DNA was separated by horizontal electrophoresis in 0.7% agarose slab gels in a Tris-borate EDTA (EDTA) buffer at room temperature at 100 volt (50 mA) for 3 h.

Pulsed-Field Gel Electrophoresis

PFGE was performed according to the standardized laboratory protocol for molecular subtyping of *Escherichia coli* by CDC (Centers for Disease Control and Prevention. 2004). To interpret the DNA fragment patterns generated by PFGE and transform them into epidemiologically useful information, the microbiologist must understand how to compare PFGE patterns and how random genetic to events can alter the patterns. A set of guidelines for interpreting DNA restriction patterns established by Tenover *et. al*., 1995.

RESULTS & DISCUSSION

Detection of virulence gene by PCR

To confirm the presence of different *E. coli* virotypes (ETEC, EPEC, EHEC, EAEC and EIEC) by PCR method; lt, st, bfpA, eae, aat and ial genes were screened respectively. None of them were EHEC and EIEC strains. Total 33 strains (among 166 samples) were positive.

Among these 18 strains (out of 71) were from diarrheal patients (CDS samples) and 15 strains (out of 95) were from non-diarrheal control (CMS samples). Among CDS samples 6 isolates were Enteroaggrigative *Escherichia coli* (11.3%), 4 isolates were Enteropathogenic *Escherichia coli* (5.6%) and 8 isolates were Entrotoxigenic *Escherichia coli* (8.5%). Among CMS samples 4 isolates were Enteroaggrigative *Escherichia coli* (4.2%), 7 isolates were Enteropathogenic *Escherichia coli* (7.4%) and 4 isolates were Enterotoxigenic *Escherichia coli* (4.2%).

Antibiotic susceptibility test

This test was performed to show whether CDS or CMS samples were resistant or sensitive to six different antimicrobial agents Ceftriaxone (CRO), Mecillinam (MEL), Ciprofloxacine (CIP), Nalidixic Acid (NA), Trimethoprim- Sulfa-Methoxazole (SXT), Amoxicillin-Clavulanic acid (AMC) which are successively representative groups of CEPHEMS (Parenteral) B, Penicillins U, Fluroquinolones B, Quinolones O, Folate Pathway Inhibittor B and β Lactam/β-Lactamase inhibitor combination.

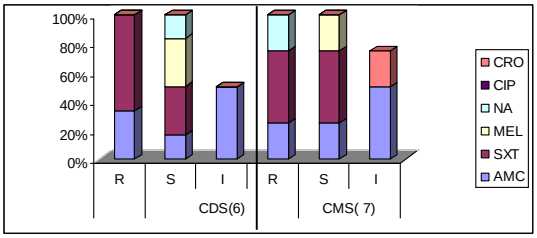

FIGURE 1: Percentage of Antibiotic susceptibility among different EAEC strains for both CDS and CMS samples.

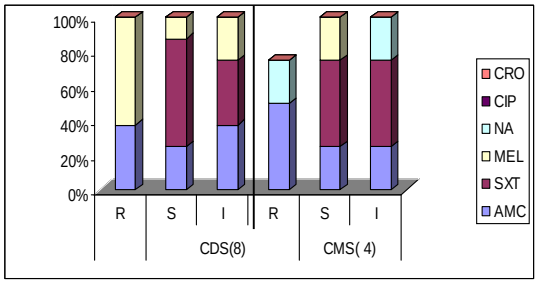

FIGURE 2: Percentage of Antibiotic susceptibility among different ETEC strains for both CDS and CMS samples.

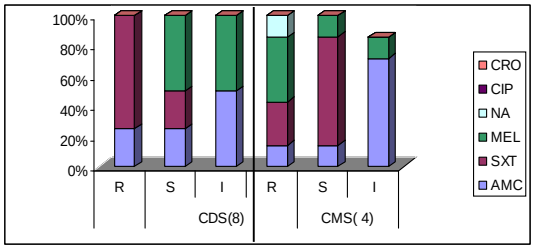

FIGURE 3: Percentage of Antibiotic susceptibility among different EPEC strains for both CDS and CMS samples.

Plasmid profile analysis

Analysis of plasmid DNA by agarose gel electrophoresis revealed that all the isolates contained multiple numbers of plasmid ranging from 1 to 140 MDa, forming different banding pattern. Middle ranged plasmid (60 MDa to 90 MDa) was found to be present in 78.78% strains. Plasmid patterns were formed according to the number and size of the plasmid and each strain was grouped into a particular pattern. In EAEC and ETEC strains for both CDS and CMS samples, all were found different plasmid pattern. Three P-1 pattern contained the plasmids of (140,60,3.7 MDa) in size in all EPEC strains. In EPEC strains another two P-2 Plasmid patterns (60, 5 MDa) were found. The remaining strains showed heterogeneous Plasmid pattern.

Pulsed-Field Gel Electrophoresis

All identified Enteroagrregative *E. coli* and Enteopathogenic *E. coli* were analyzed by PFGE. PFGE was performed by the standardized protocol developed by CDC for *E. coli* (CDC protocol, 2004). PFGE analysis of Xba-I digested chromosomal DNA of the strains yielded 12 to 16 reproducible DNA fragments ranging in size approximately from 30 to 600Kb (Fig. 4 and 6). Analysis of the TIFF images was carried out by the BioNumerics software package (Applied Maths, Belgium) using the Dice coefficient. Banding profile analysis of the chromosomal DNA based on Tenovar theory (Tenover *et al.*, 1995) suggested that all the strains of EAEC were grouped into eleven types (designated as A, B, C, D, E, F, G, H, I, J, K) (Fig. 4). Among these, no similarity was found. However, the banding patterns of almost all EAEC strains were completely different. In case of all EPEC strains 3 strains were clonally homologue (type A) and rest were clonally diverse (type B, C, D, E, F, G, H, I) (Fig 6). Dendrogram analysis of all EAEC strains showed that they were heterogeneous. In case of all EPEC strains, Dendrogram analysis showed that 3 strains were clonally homolog where 2 strains came from non-diarrheal controls and another 1 from diarrheal patients (Fig. 7).

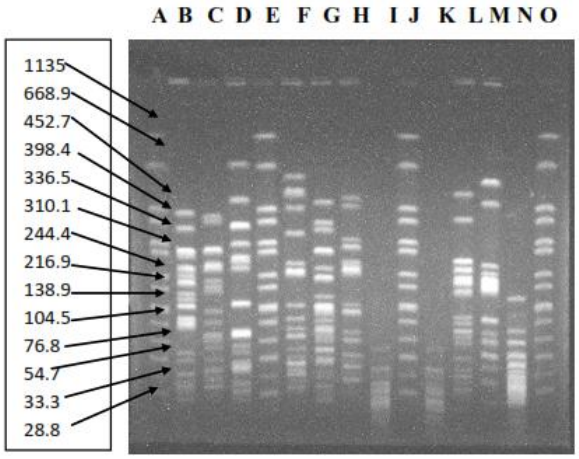

FIGURE 4: PFGE banding patterns of XbaI-digested chromosomal DNA of representative strains of Enteroaggregative *E. coli*. Lanes: A: Salmonella Branderup H9812 (marker); B: 7212 CMS-3 C: 7207 CMS-5; D: 7160 CMS-7 ; E: Salmonella Branderup H9812 (marker); F: 8070 CMS-1; G: 8016 CDS-3 ; H: 7136 CDS-4 I: 7191 CDS-1 ; J: Salmonella Branderup H9812 (marker); K: 8334 CDS-1; L: 7207 CDS-2 ; M: 7213 CDS-2 ; O: Salmonella Branderup H9812 (marker).

According to criteria published by Tenover *et al.*, 1995, the isolates were analyzed. More than 6 bands differences between two strains have been considered as different PFGE pattern. In this case, Xba I enzyme was used as, it was recommended by the PulseNet program. It gave the best discrimination of the strains, since it has a long

ranged DNA cutting site and cut the DNA infrequently. Infectious diarrheal diseases contribute a considerable problem globally and are responsible for considerable morbidity and mortality, especially in the developing countries (WHO 1993). Diarrhea also remains an important problem in the industrialized countries, but the course of the disease is generally mild, and the mortality has decreased drastically over time (Mark et al., 2016). Moreover, the numbers of diarrheal outbreaks have

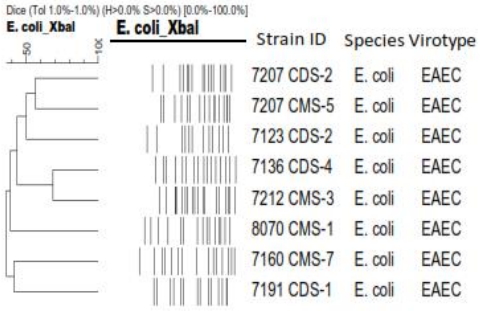

FIGURE 5: Dendrogram showing the PFGE patterns of EAEC strains isolated from diarrheal patients and non-diarrheal controls. The dendrogram was constructed with Bionumerics 4.5 software using the unweighted pair group method with arithmetic means (UPGMA) method. Thin lines were added by the program and show the location (peak of densitometry curve) of less-intense bands included in the analysis.

Strain ID	Plasmid Pattern	Antibiotic Susceptibility Profile	PFGE Pattern
7207 CDS-2	P3	$AMC^{I}SXT^{R}MEL^{S}NA^{R}CIP^{I}CRO^{S}$	A
7207 CMS-5	P10	$AMC^{I}SXT^{R}MEL^{S}NA^{R}CIP^{S}CRO^{S}$	B
7123 CDS-2	P8	$AMC^{S}SXT^{S}MEL^{S}NA^{S}CIP^{S}CRO^{R}$	C
17136 CDS-4	P5	$AMC^{R}SXT^{R}MEL^{S}NA^{R}CIP^{S}CRO^{S}$	D
7212 CMS-3	P12	$AMC^{R}SXT^{S}MEL^{S}NA^{S}CIP^{S}CRO^{S}$	E
8070 CMS-1	P9	$AMC^{S}SXT^{S}ME^{S}NA^{R}CIP^{S}CRO^{S}$	F
7160 CMS-7	P11	$AMC^{I}SXT^{R}MEL^{S}NA^{R}CIP^{S}CRO^{S}$	G
7191 CDS-1	P4	$AMC^{I}SXT^{R}MEL^{I}NA^{R}CIP^{S}CRO^{S}$	H

TABLE 1: Summary of all characteristic patterns of EAEC strains Key: I=Intermediate, R=Resistant, S=Sensitivity

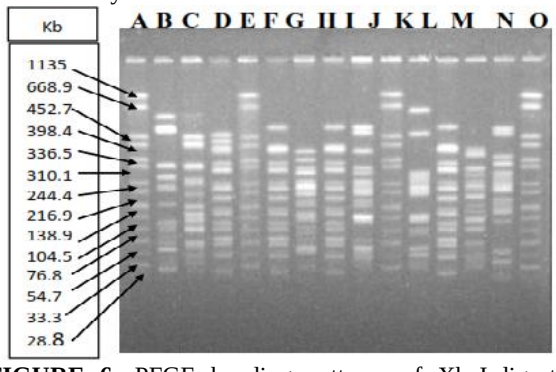

FIGURE 6: PFGE banding patterns of XbaI-digested chromosomal DNA of representative strains of Enteropathogenic E.Coli. Lanes: A: Salmonella Branderup H9812 (marker); B: 7121 CDS-4 C: 7071 CDS-5; D: 7108 CDS-7; E: Salmonella Branderup H9812 (marker); F: 8027 CDS-1; G: 7175 CMS-7 ; H: 7108 CMS-9 I: 8001 CMS-5 ; J: Salmonella Branderup H9812 (marker); K: 7012 CMS-13; L: 7211 CMS-5 ; M: 7166 CMS-27; N: 7176 CMS-7 O: *Salmonella Branderup* H9812 (marker).

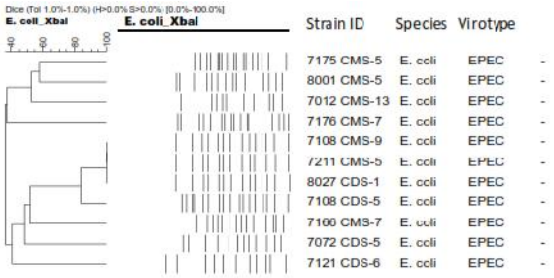

FIGURE 7: Dendrogram showing the PFGE patterns of EPEC strains isolated from diarrheal patients and non-diarrheal controls. The dendrogram was constructed with Bionumerics 4.5 software using the unweighted pair group method with arithmetic means (UPGMA) method. Thin lines were added by the program and show the location (peak of densitometry curve) of less-intense bands included in the analysis

Strain ID	Antibiotic Susceptibility Profile	Plasmid Pattern	PFGE pattern
7175 CMS-5	$AMC^{I}SXT^{S}MEL^{S}NA^{S}CIP^{S}CRO^{S}$	P15	B
8001 CMS-5	$AMC^{I}SXT^{S}MEL^{S}NA^{S}CIP^{S}CRO^{S}$	P2	C
7012 CMS-13	$AMC^{R}SXT^{S}MEL^{R}NA^{R}CIP^{S}CRO^{S}$	P20	D
7176 CMS-7	$AMC^{I}SXT^{S}MEL^{I}NA^{R}CIP^{S}CRO^{S}$	P18	E
7108 CMS-9	$AMC^{I}SXT^{R}MEL^{S}NA^{R}CIP^{S}CRO^{S}$	P1	A1
7211 CMS-5	$AMC^{I}SXT^{R}MEL^{S}NA^{R}CIP^{S}CRO^{S}$	P1	A1
8027 CDS-1	$AMC^{I}SXT^{R}MEL^{I}NA^{R}CIP^{S}CRO^{S}$	P1	A1
7108 CDS-5	$AMC^{S}SXT^{S}MEL^{S}NA^{R}\ CIP^{S}CRO^{I}$	P16	A2
7166 CDS-5	$AMC^{R}SXT^{S}MEL^{R}NA^{R}CIP^{S}CRO^{S}$	P17	G
7072 CDS-5	$AMC^{R}SXT^{R}MEL^{R}NA^{R}CIP^{R}CRO^{R}$	P24	H
7121 CDS-6	$AMC^{I}SXT^{R}MEL^{R}NA^{R}CIP^{S}CRO^{S}$	P14	F

TABLE 2: Summary of all characteristic patterns of all EPEC strains. Key: I=Intermediate, R=Resistant, S=Sensitivity increased during recent years in the industrialized countries (Armstrong *et al.* 1996). Recently it was concluded that 20% of Britons had infectious intestinal disease each year (Wheele *et al.* 1999). Results presented in different figures and tables showed the phenotypic and molecular characterization of the EPEC, EAEC and ETEC strains from diarrheal patients and non-diarrheal controls were performed. This study substantiated the study of previous works. In Bangladesh, many infants and young children develop diarrhea due to infection with *E. coli* (Faruque; 1992). Previous studies mainly focused on *E. coli* strains of clinical origin commonly belonging to the ETEC, EPEC and EAEC categories (Albert, 2000, Faruque, 1992). Molecular methods, especially PCR, are nowadays considered the most reliable and sensitive techniques for differentiating diarrheagenic *E. coli* strains from nonpathogenic members of the stool flora, and for distinguishing one *E. coli* pathogroup from another (Nataro and Kaper, 1998). In this study, PCR was used for detecting different virotypes of *E. coli*. The *E. coli* isolates were tested for the presence of the following virulence genes by multiplex PCR: *eaeA* (*E. coli*

attaching and effacing), *bfpA* (bundle-forming pilus), *aat* (EAEC heat stable enterotoxin) and lt (heat labile) and st (heat stable) enterotoxin. At the same time the isolates were also screened for *ial* gene by PCR. Several other works including Keiko kimata *et al*, 2005; Pass *et a*l, 2000; Rappelli *et al*, 2001 have got similar results. They used several other genes in their experiments. However they used only in diarrheagenic patients. This study has been extended to non-diarrheagenic patients. Results presented in the fig. 1, 2 and 3 showed that among CDS samples 6 isolates were Enteroaggrigative *Escherichia coli* (*aat* gene), 4 isolates were Enteropathogenic *Escherichia coli* (*bfp, eae* genes) and 8 isolates were Entrotoxigenic *Escherichia coli* (*lt,st* genes) . Among CMS samples 4 isolates were Enteroaggrigative *Escherichia coli* (*aat* gene), 7 isolates were Enteropathogenic *Escherichia coli* (*bfp, eae* genes) and 4 isolates were Enterotoxigenic *Escherichia coli* (*lt,st* genes). This result showed that anyone of the genes or all the genes were responsible for diarrhea due to *E. coli*. The irrational and inappropriate use of antimicrobial agents facilitates the emergence of drug resistance in Bangladesh like other developing countries. Acute or chronic enteritis due to the different categories of *E. coli*, mainly EPEC and EAEC, is an emerging problem in many parts of the world (Nataro, J. P., and J. B. Kaper**.** 1998). It has been estimated that 9.2 million deaths in the developing world have been caused by infectious diseases, and diarrheal diseases are the fourth most prevalent cause (Murray, *et al.,* 1997). Most mild diarrhea cases are successfully managed with oral rehydration therapy. Only for more severe or persistent diarrhea cases, antimicrobial treatment should be added. Ampicillin and cotrimoxazole have been recommended by the World Health Organization. Since antibiotic resistance is a major phenotypic trait particularly for the clinical isolates it has a potential interest in exploring the characteristics of these EPEC, EAEC and ETEC isolates. In the present study, the susceptibility test results presented in table 1 and 2 showed that most of the *E. coli* strains are multidrug resistant. In the diarrheal patients, most of the EAEC strains were Nalidixic Acid, Mecillinam and Trimithoprim Sulphomithoxasol resistant whereas in the non-diarrheal control, they were resistant to Nalidixic Acid and Trimithoprim Sulphomithoxasol. Most of the ETEC strains are Nalidixic Acid, Mecillinam, Amoxicillin and Trimithoprim Sulphomithoxasol resistant whereas in the non-diarrheal control, they were resistant to Nalidixic Acid and Trimithoprim Sulphomithoxasol. In case of EPEC strains of diarrheal patients , most strains were resistant to Trimithoprim Sulphomithoxasol and Nalidixic Acid, but strains of non-diarrheal controls were resistant to Nalidixic Acid and Mecillinam. Of all the 33 strains, 3 were resistant to Ciprofloxacin (10%). The risk of antibiotic resistance is becoming alarming in the country. In near future this may be a life threatening for the population. Public awareness must be created non-judicious use of antibiotics. Analyses of plasmid profiles are useful tools with which to document the appearance of plasmid associated with important phenotypic characteristics. Electropherotyping of plasmid DNA of different EPEC, EAEC and ETEC strains might be useful to determine the clonal diversity among the strains. The plasmid pattern was very diverse in both ETEC and EAEC strains. Analysis of the plasmid DNA of *E. coli* isolates had shown that all isolates contained multiple numbers of plasmid ranging from 1.0 to 140 MDa. based on number and size of plasmids. A significant association may exist between the presences of small plasmids and ecology and/ or pathogenicity of the isolates. The presence of additional plasmids in patterns suggests that plasmid profiles may be useful in distinguishing between different *E. coli* strains. It may also be possible to document the appearance of any new strains in a community by these patterns (Haider *et al.*, 1989).An analysis of the plasmid DNA of EPEC, ETEC and EAEC strains has shown that in 11 EPEC strains most of the plasmid size varies from 60 MDa to 140 MDa. Three isolates showed identical plasmid profile (140,60,3.7 MDa) and another two isolates also showed another identical plasmid pattern (60,5.0 MDa). Rest of the EPEC strains showed heterogeneous plasmid profile. Baldini *et al.* 1983 showed that the ability of EPEC strain to adhere in a localized pattern was dependent on the presence of a 60-MDa plasmid (Baldini. *et al*., 1983). This plasmid was therefore designated as EPEC adherence factor (EAF) plasmid (Baldini, *et al*., 1986). The genes for AAF/I are organized as two separate gene clusters on the 60-MDa plasmid of strain 17-2, separated by 9 kb of intervening DNA (Savarino, *et al*.,1994).In this study, the plasmids of similar sizes were also detected in most of the EPEC, ETEC and EAEC isolates. Therefore, this size-ranged plasmid could be used as a molecular marker for *E. coli* strains' identification. Plasmids are responsible for transfer of drug resistance to other organisms in hospital and community. A discriminatory, reproducible and recently evaluated molecular typing system called pulsed-field gel electrophoresis (PFGE) has been used for the determination of clonal relationships among the *E. coli* isolates. In this method, chromosomal DNA is digested with a restriction endonuclease that generates large fragments (Chung *et. al.*, 2000). The restriction fragments are resolved into a pattern of discrete bands in an agarose gel in which orientation of the electric field across the gel is changed periodically ('pulsed') rather than kept constant as in conventional agarose gel electrophoresis. The DNA restriction patterns of the isolates are than compared with one another to determine their relatedness. Choice of restriction enzyme is an important factor to obtain a reproducible and well discriminatory banding pattern in PFGE. The banding patterns were analyzed by Bionumerics. In this case, Xba I enzyme was used as, it was recommended by the PulseNet program. It gave the best discrimination of the strains, since it has a long ranged DNA cutting site and cut the DNA infrequently. After analyzing PFGE pattern, it was found that all EAEC strains were clonally diverse. All these strains also showed different plasmid profiles. Performing PFGE pattern analysis of all EPEC strains, 3 strains were clonally homologue and it was also confirmed by dendrogram analysis (Fig. 7). These three strains had also similar plasmid pattern and antibiotic susceptibility profile. It is shown table 1 and 2 where one strain came from diarrheal patient and another two strains came from diarrheal controls.

CONCLUSION

Resistance to antibiotics is very common in bacterial isolates worldwide. This study will provide information for antibiotic therapy and resistance control for health professionals. Besides this study concerns us about the pathogenicity of EPEC. An actively flourished bacterial strain(s) found in non-diarrheal control may not show their pathogenicity although having same genotypic and phenotypic characteristic of diarrhea causing EPEC strain.

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