Incidence and Molecular Analysis of Entero Toxigenic *Escherichia coli* causing Diarrhea among Children in Odisha, India 2006

Hemant Kumar Khuntia, Surya Kanta Samal, Alakananda Nayak, Santanu Kumar Kar and Bibhuti Bhusan Pal*

Regional Medical Research Centre (ICMR), Chandrasekharpur, Bhubaneswar - 751 023, India.

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Hospital based study was established in Odisha, India to understand the epidemiology of diarrhoeal disease among children. During the year 2006 samples were collected from hospitalized children less than 14 year of age who had diarrhea. Colonies from each culture with morphology typical of that of *Escherichia coli* were tested for the presence of *elt* gene encoding heat labile enterotoxin by PCR assay. Of the presumptive *E.coli* isolated from 151 children from a total of 250 diarrhoeal children, 7% (11/151) were found entero toxigenic *E.coli* (ETEC) carrying *elt* gene. The antibiotic susceptibility pattern of ETEC was ciprofloxacin, norfloxacin, chloramphenicol, gentamycin and tetracycline. RAPD DNA finger printing analysis revealed genetic homogeneity among our ETEC strains. ETEC is one of important enteric pathogens causing diarrhea among children in Odisha which should be closely monitored.

Key words: Diarrhea, *E. coli*, ETEC, elt, Antibiotics, RAPD and Clonality.

Diarrhoeal disorder continue to be a major health problem world wide[1-2] especially in developing countries where they are estimated to be responsible for 2-5 million infant deaths per year with annual mortality rate of 4.9 per 1000 children and incidence of 3.2 episode per child per year among children under 65 years of age[2]. Diarrhoeagenic *Escherichia coli* pathotypes (DEPS) represent a leading bacterial cause of paediatric diarrhea in developing regions[3]. The DEPS that cause diarrhea include entero pathogenic *E.coli* (EPEC), entero toxigenic *E. coli* (ETEC), enteroinvasive *E.coli* (EIEC), shigha toxin producing *E.coli* (STEC)[4,3]. These pathogens are defined by the presence of one or more definable *E.coli* virulence factor.

Orissa an eastern state of India has been documented to be worst affected by diarrhoeal disorder leading to high morbidity and mortality[5] followed by monsoon break, cyclone and flood. Regional Medical Research Center (RMRC), Bhubaneswar, started research studies on diarrhoeal disorder since 1995 to document the spectrum of etiology of diarrhoeal disorders. Since then RMRC continued several hospital based and outbreak studies in the state and established a

* To whom all correspondence should be addressed.
Tel.: +91-674-230138; Fax: +91-674-230132
E-mail: bbpal_rmrc@yahoo.co.in

surveillance unit at infectious disease hospital at Puri to document diarrhea causing bacterial pathogens. The investigations since 1995 revealed that toxigenic *Vibrio cholerae* of different serogroups and biotypes was reported as the sole pathogen causing cholera like illness followed by toxigenic *E.coli*[6-8]. Here we report the incidence of ETEC associated with hospitalized children with acute diarrhoea in Odisha and their genetic analysis during 2006.

MATERIAL AND METHODS

Bacteriological analysis

During the period in 2006, 250 rectal swabs were collected from freshly admitted cases having acute diarrhoeal symptoms from Capital hospital (Bhubaneswar), Sishu bhaban (Cuttack), District hospital (Puri) and Infectious disease hospital (Puri), before any treatment was initiated by the government health authorities. Rectal swabs in Cary Blair transport medium (CBT) were transported to the Regional Medical Research Center (RMRC) laboratory for bacteriological analysis to isolate *V.cholerae* and other enteropathogens such as *E.coli* spp*, Shigella* spp. and S*almonella* spp. by previously published technique[9-10]. Diarrhoea is defined as three or more watery or loose stools in a 24-hour period prior to admission to the hospital. At least three lactose fermenting colonies from the MacConkey agar plate were tested individually on Triple sugar iron agar (TSI) agar for presumptive identification of *E. coli* spp.

Antimicrobial susceptibility

Antimicrobial sensitivity pattern was tested for isolated ETEC strains confirmed by PCR assay with specific primer pair following the Kirby-Bauer method[11] with the antibiotics (Hi-media Laboratories, Bombay, India) ampicillin (A, 10µg), chloramphenicol (C, 30µg), co-trimoxazole (Co, 25µg), ciprofloxacin (cf, 5µg), furazolidone (Fr, 100µg), gentamicin (G, 10µg), neomycin (N, 30g), nalidixic acid (Na, 30µg), norfloxacin (Nx, 10µg), streptomycin (S, 10µg) and tetracycline (T, 30µg). Characterization of strains as susceptible, intermediate or resistant was determined based on the size of the inhibition zone around each disc according to manufacturer's instruction. Strains showing an intermediate zone of inhibition were interpreted as resistant to that drug on the basis of previous minimum inhibition of concentration (MIC) studies conducted with ETEC[12].

Polymerase Chain Reaction (PCR) assay for identification ETEC

E.coli strains were screened for the presence of gene, *elt* gene (encoding heat -labile toxin) for ETEC, using specific primers as described earlier[13]. Template DNA was prepared from culture grown in Luria Bertani broth for over night by boiling in a water bath for 10 min and instantly cooling on ice. PCR amplification was done with appropriate volume of 10x amplification buffer (500mM KCl, 100mM Tris-HC, 15mM $MgCl2$, [P^{H}-8.3]), 2.5 mM each deoxynucleoside triphosphate, 10 pmol of each primer, 1.25 unit of Taq DNA polymerase (Bangalore Genei, India), and 5µl of template DNA. The reaction volume was adjusted to 25µl using sterile triple distilled water. Simplex PCR assay was performed in an automated thermo-cycler (G-Storm, England) for 30 cycles using condition described earlier[13]. Aliquots of PCR product analyzed by agarose (1.8% wt/vol) gel electrophoresis in Tris-borate EDTA buffer stained in ethidium bromide and visualized under UV in Alpha Imager (Alpha Infotech Corporation, USA).

Randomly Amplified Polymorphic DNA (RAPD) assay

RAPD was performed using the short oligonucleotide 1281 (5-AACGCGCAAC-3) as described earlier[7]. The amplicons were electrophoresed in 2% (w/vol) agarose at 80V for 45 min and stained in ethidium bromide. The DNA fingerprinting pattern was visualized under UV light.

RESULTS

Two hundred and fifty rectal swabs collected during the study period were bacteriologically analyzed using standard methodology. The analysis revealed 151 (60.4%) were culture positive for *E. coli* spp., followed by *V. cholerae* (0.67%) and Shigella spp. (3.9%). Initially PCR assay of 50 representative isolates of *E.coli* spp. using specific primer pair revealed 3 (6.0%) were positive for *elt* gene (Fig1) indicating the association of ETEC causing diarrhea. Further retrospective PCR analysis of 50 *E.coli* spp. from the laboratory stock revealed 4 (8.0%) was positive for *elt* gene. All together the results revealed 7%

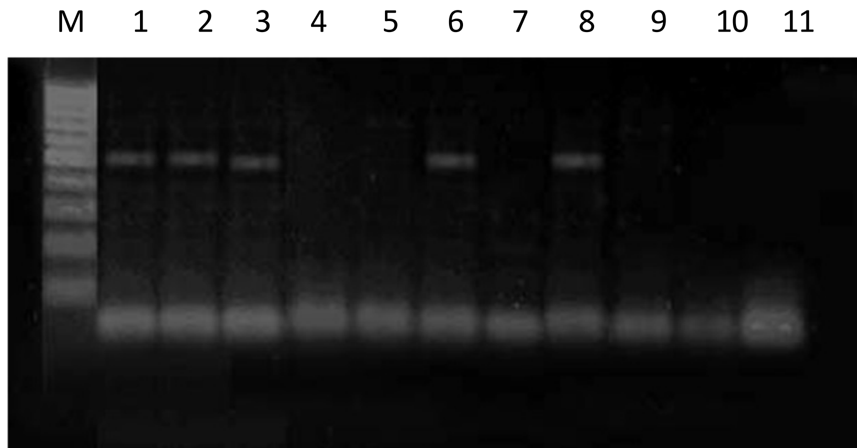

Fig. 1. Ethidium Bromide agarose gel electrophoresis of simplex PCR detects elt gene of ETEC. Lane M, 100 bp DNA Ladder (Bangalore Genei), Lane 1, ETEC, Control strain Kolkata (Positive for *elt* gene), Lane 2, CH 521 (Positive for *elt* gene), Lane 3, CH 520 (Positive for *elt* gene), Lane 4, CH 532 (Negative for *elt* gene), Lane 5, CH 534 (Negative for *elt* gene), Lane 6, CH 515 (Positive for *elt* gene), Lane 7, PU 1112 (Negative for *elt* gene), Lane 8, PU 1175 (Positive for *elt* gene), Lane 9, CH 542 (Negative for *elt* gene) and Lane 10, CH 516 (Negative for *elt* gene)

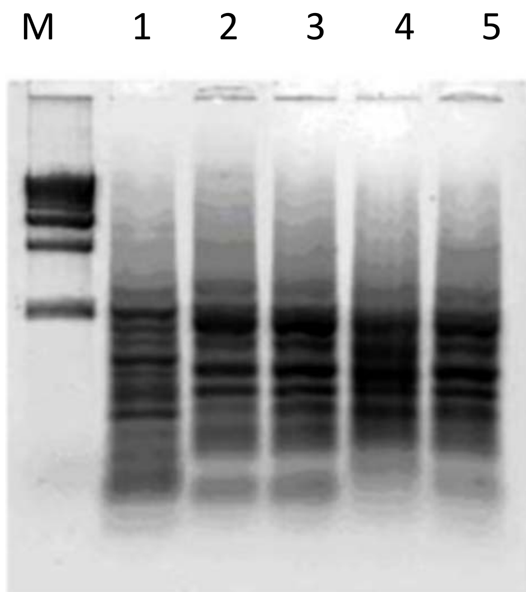

Fig. 2. Ethidium Bromide agarose gel electrophoresis of RAPD PCR using 1281 primer. Lane M, 1Kb DNA Ladder (Bangalore Genei), Lane1, ETEC, Control strain Kolkata (Positive for *elt* gene), Lane 2, H 521 (Positive for *elt* gene), Lane 3, CH 520 (Positive for *elt* gene), Lane 4, CH 515 (Positive for *elt* gene), Lane 5, PU 1175 (Positive for *elt* gene)

ETEC were responsible for causing diarrhea among children. Of the total 250 hospitalized children with acute diarrhea, highest 150 (60%) cases belonged to <2 years age groups. The common antibiotic susceptibility pattern was CcfGNxT while resistance profile was ACoNaSFr. Considering the number and location of the RAPD DNA finger printing bands of our ETEC strains and standard strain of Kolkata (Fig 2), the analysis demonstrated existence of genetic homogeneity among our ETEC strains as well as similar to the Kolkata strain.

DISCUSSION

Prior to implementation of control measures for ETEC diarrhea it is logical to assess the incidence, antibiogram pattern and genotypes of the bacterium. In this study we describe the incidence of ETEC among the hospitalized patient's belonged to pediatric age groups, antibiogram pattern of ETEC isolates, association of different toxic genes and clonality.

The rates of recovery of ETEC reported in this study are more or less very similar to those hospital based studies of ETEC in northern Egypt and Indonesia[14-17]. Despite the other diarrhoeagenic bacterial pathogens, 7% of the

hospitalized acute diarrhea patients were contributed by ETEC. ETEC associated diarrhoeal infant patients were found with clinical manifestation of profuse watery diarrhea with little or no fever, vomiting and dehydration. Superiority of ETEC diarrhoea may be due to the interaction of colonization factor followed by heat-labile toxin (LT) or heat stable toxin (ST) or both the toxin by bacterial disrupts in intestinal epithelial cells signaling pathway in the host resulting in diarrhea. The elaboration of severe diarrhea may be the result of immunologically naive child's first encounter with ETEC pathogen.

In our study peak of ETEC infection were encountered in rainy season followed by winter and summer in contrast to the report in Mexico[18] and Egypt[19]. Overall, our results suggest that humidity during the rainy season may be an important factor for ETEC spread and disease in Odisha.

E.coli pathovars are diverse microorganisms composed of several clones the genetic back ground of each of which encodes distinct combination of virulence determinants. Accordingly use of conventional classification of *E.coli* by O antigen and H-antigen serotype may restrict our appreciation of the genetic relationship of different diarrhoeagenic *E.coli* pathotypes[20-23]. Phenotypic and genotypic heterogeneity has been demonstrated among ETEC isolates of similar serogroups[24]. The genetic analysis data obtained in this study by employing RAPD revealed the existence of genetic homogeneity among our strains and Kolkata strain indicating the circulation of unit clonality carrying LT. However the selective immune pressure on ETEC pathogenecity from a host is unknown. In view of this statement further study is needed to obtain a better understanding of the naturally acquired ETEC anti-toxin immune response.

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