Multidrug Resistant Enteropathogenic *E.Coli* Diarrhea in Children

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Abstract

Diarrhea is an important cause of morbidity and mortality in developing countries like India. Studies focusing on individual bacterial agents causing pediatric diarrhea published in recent times, are few and far between. This paper focuses on hospitalized children with acute diarrhea with special reference to enteropathogenic *E.coli* (EPEC), presenting to a tertiary level teaching hospital. Four hundred children were enrolled into the study. All admitted children, were treated as per the WHO mandated protocol for management of acute diarrhea, with oral rehydration solution, intravenous fluids (wherever clinically indicated), oral zinc suspensions (at 20 mg/day for 14 days for children > 6months and 10 mg/day for 14 days for children < 6 months). Out of 400 children, with acute diarrhea, EPEC was detected in 40 (10%) cases. Colonies agglutinating with polyvalents 1, 2 and 3 were labeled as Enteropathogenic *E.coli*, as per the kit manufacturer's criteria. Subsequently using monovalent antisera specific O type was determined. The mean age of patients was12 months (range 2 months to 36 months) and the male female ratio was 1.85.Out of 40 children, 32(80%) had 6 to 24 stools per day. Vomiting was seen in 28 (70%) children. 31 children (77.5%) had fever at presentation. Dehydration was seen in 19(47.55%) cases. 28 children (70%) had anemia (hemoglobin less than 10 gm %). As regards nutritional status, 21 children (52.5%) were underweight or severely underweight. Stunting was seen in 11 (32.5%) cases. Some degree of wasting was seen in 17(42.5%) children. Molecular typing using gene specific primers revealed that the strains were typical EPEC harbouring eaea

and bfp genes. The resistance patterns to various antibiotics were as follows: Nalidixic acid 95%, Amoxicillin 90%, Cefotaxime 77.5%, Norfloxacin 77.5%, Ceftriaxone 75%, Ciprofloxacin 72.5%, Ofloxacin 70%, Nitrofurantoin 27.5%, Azithromycin 25%, Gentamicin 17.5%, and Amikacin 12.5%.The widespread resistance to ciprofloxacin and ceftriaxone is alarming as they are the first line antimicrobials recommended by WHO. Gentamicin and Amikacin are emerging as alternate useful drugs. This is the first detailed study of clinical spectrum , detailed molecular typing and antibiotic resistance patterns of enteropathogenic E.Coli diarrhea from India in recent times.

Key words: diarrhea , enteropathogenic E.coli, antibiotic resistance

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Introduction

Diarrhea remains the second leading cause of death in children younger than 5 years globally [1]. Of the 4.879 million global deaths of children, below 5 years of age due to infectious diseases, diarrhea alone has caused 0.801 million deaths in 2010. Of India's more than 2.3 million annual deaths among children, about 334,000 are attributable to diarrheal diseases [2]. This large burden of disease continues, despite improvements like widespread availability of oral rehydration solution, antibiotics, clean water, improved sanitation, and breast-feeding. [3, 4].

In order to decrease the diarrheal diseases burden, the etiology of diarrhea must be understood to accelerate additional preventive measures. Diarrhea can be caused by bacterial, viral and parasitic pathogens.

Most studies have focused on specific etiologic agents. Among children below five years of age, Diarrheagenic *E. coli* (DEC), such as Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli*

(EPEC), Enteroaggregative *E. coli* (EAEC), are the most important enteric pathogens, responsible for 30 to 40% of all the diarrheal episodes in developing countries [5,6].

Enteropathogenic *Escherichia coli* (EPEC), one of the diarrheagenic *E. coli* pathotypes, are among the most important pathogens infecting children worldwide, because of their high prevalence in both the community and hospital setting [7]. They are also one of the main causes of persistent diarrhea [8].

Published literature on acute diarrhea in Indian children, elaborating the clinical features and antibiotic spectrum of enteropathogenic *E.coli* diarrhea in recent times is scanty. Our interest was to investigate the prevalence of the enteropathogenic *E. coli* in clinically relevant (i.e., hospitalization-requiring) childhood acute gastroenteritis and to do molecular studies and antibiotic sensitivity patterns of these isolates. Children with persistent diarrhea were not included as our focus was on acute diarrhea cases presenting to this tertiary care hospital catering to Northern India. Our focus was primarily acute diarrhea due to enteropathogenic E.*coli.*

Materials and Methods

Written informed consent, was obtained from the parent or guardian of the child before recruitment, and the study was approved by the Ethical Committee of Maulana Azad Medical College, New Delhi, India vide number F.1/IEC/MAMC/(36)/2/2013/No 109 dated 21/5/2013. The written informed conscent form was signed by parents in English or Hindi.

Sample size calculation.

The formula for sample size calculation used was estimating a population proportion with specified precision.

The formula used was : $n = \frac{(za/2)^2\, p(1-p)}{d^2}$

(1) p= percentage of prevalence

(2) d= difference in percentage from the actual prevalence.

(3) The chance percentage of (2).

For large n, the distribution of small p can be approximated by Gaussian form. In (3) let the chance percentage be atleast 95%. The tolerable least confidence in the estimate is denoted by a. Therefore a=5%=5/100=0.05. For a= 0.05, za/2 =1.96(for a normal distribution) for 2 sided CI. Let prevalence p=20%=20/100=0.2. Let d be 4%=4/100=0.04.

Taking a=0.05, za/2=1.96, d=0.04, p=0.2

$N = \frac{(1.96)^2 x (0.2) x (1-0.2)}{(0.04)^2} = 384.$ Hence a sample size of 400 was chosen.

Inclusion criteria

1. Children less than 12 years age with acute diarrhea defined as diarrhea of less than 7 days duration admitted to diarrhea ward of Lok Nayak hospital were enrolled in the study.

2. Children should not have received any antibiotics for the current illness.

Exclusion criteria

Children with ileostomy and colostomy diarrhea were excluded from the study.

The children were enrolled between March 2014 and June 2014 as this is the diarrheal season in New Delhi (India). The chance of isolating Enteropathogenic E.coli is maximum during this season.Between March 2014 and June 2014, a total of 400 stool samples from children with acute diarrhea, admitted to the diarrhea ward of Lok Nayak Hospital, attached to Maulana Azad Medical College, a major tertiary level teaching hospital, was analyzed for various bacterial enteropathogens. Acute diarrhea is defined as passage of 3 or more watery stools, over a 24 hour period and lasting less than 14 days, as mandated by the WHO [9].

After obtaining informed written conscent (patient information sheet and conscent forms in Hindi and English were developed) a detailed history was obtained with a predesigned proforma

.At enrolment, each infant underwent a clinical examination and parents were interviewed to obtain data on gestational age, kind of delivery (spontaneous or caesarean), birth weight, type of feeding (exclusively or partial breast-feeding or formula-feeding), details of loose stools, vomiting and fever. The data was captured on a pre-designed proforma. A detailed clinical examination was also conducted and the weight, length/height measurements were recorded as per standard methodology. Freshly passed stool samples were collected in clean sterile wide mouthed containers and the sample transported to microbiology laboratory within 2 hours of collection. Stool samples of children presenting to hospital between 8am to 9 am daily were enrolled so that freshly passed stool specimen could be transported to the laboratory for processing.

The children had not received any antibiotic therapy in the week preceding the sampling. About 5-10 grams faeces were collected from each patient. Each sample, was stored at in a numbered screw-capped plastic container in aerobic conditions and was processed within 2 hours of collection.

All admitted children, were treated as per the WHO mandated protocol for management of acute diarrhea, with oral rehydration solution, intravenous fluids (wherever clinically indicated), oral zinc suspensions (at 20 mg/day for 14 days for children > 6months and 10 mg/day for 14 days for children < 6 months) and antibiotics [10]. Children were administered intravenous ceftriaxone in cases of dysentery. In case of no response injection amikacin was added after 48 hours. Antibiotics were continued for 5 days. Hemoglobin, total leukocyte counts, serum electrolytes and kidney function tests (blood Urea and serum creatinine), were carried out in all patients. A blood gas analysis was also done at admission to detect metabolic acidosis. Electrolyte imbalances were treated as per standard treatment guidelines. All children were admitted till the complete subsidence of diarrhea.

Age appropriate feeding advice was recommended by the dietician, and those who were severely underweight and/or severely wasted, were treated for severe malnutrition. Vitamin and mineral supplementation, were given to those wherever clinically indicated. Oral zinc supplementation was also prescribed for all children during the period of hospitalization, and continued after discharge to complete a 14 day therapy.

Stool Examination for Enteric Pathogens

Stool specimens were collected from the patients in clear, transparent, wide-mouthed bottles and transported to the Enterobacteriaceae Laboratory, of the Dept. of Microbiology, Maulana Azad Medical College. The children were provided sterile plastic sheets and liquid stools were collected directly after passage. The specimens were examined grossly for consistency, color, and atypical components such as mucus, blood, and parasites. The specimens were also examined by light microscope for the presence of red blood cells, pus cells, parasitic ova, and protozoa.

The stool samples were cultured on MacConkey agar, Xylose Lysine Deoxycholate agar and Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar. They were incubated at 37 °C overnight. Samples were also inoculated in enrichment media, Selenite F Broth, and alkaline peptone water for *Salmonella* and *Vibrio* spp., and sub-cultured onto MacConkey agar and TCBS agar, respectively. They were identified by standard biochemical methods and sero-grouping.

All samples from hospitalized patients, were examined for other enteric bacterial pathogens, in addition to potential pathogenic *E. coli*, using standard microbiology procedures (11). For isolation of *E. coli*, stool specimens were plated on MacConkey (Hi Media), followed by incubation for 16–18 hrs at 37°C. Five typical, lactose fermenting pink colour colonies per sample, were selected confirmed as E.*coli* by their motility and standard biochemical reactions namely indole-production ,negative for urease hydrolysis and citrate utilization.Viral isolations were not attempted in this study.

For identification of EPEC, slide agglutination with antisera to common EPEC O antigens, was carried out. *E. coli* strain grown on a nutrient agar plate, was suspended in normal saline solution, autoclaved for 15 minutes and then examined by slide agglutination using commercially available antisera, in a kit identified as “Pathogenic *E. coli* Antisera” (Denka Seiken Co.,Ltd.,Tokyo,Japan). Colonies agglutinating with polyvalents 1, 2 and 3 were labeled as Enteropathogenic *E.coli*, as per the kit manufacturer’s criteria. Subsequently using monovalent antisera specific O type was determined. This was also in accordance with these serogroups being pathogenic as published earlier by Tamaki Y et al [12]. As per Tamaki Y et al, the likelihood of enteropathogenic *E. coli* in other polyvalent sera namely P4, P5, P6, P7 and P8 is very less likely.

Antimicrobial Susceptibility Tests

All EPEC strains, were analyzed for their antimicrobial susceptibility pattern by the Kirby–Bauer disc diffusion method. Antimicrobial drug susceptibility testing, was carried out using standard methods (disc diffusion method) using Mueller-Hinton agar, according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2009) [13]. Antibiotic discs(HiMedia), amikacin (amika 30 mcg), azithromycin (azithro 15 mcg), amoxicillin (amoxy 10mcg) cefotaxime (cefotax 30mcg),ceftriaxone (ceftriax 30 mcg), ciprofloxacin (cipro 5mcg),gentamicin (genta 10 mcg) ,nalidixic acid (nalidixic acid 30mcg),nitrofurantoin (nitrofuran 300mcg), norfloxacin(norflox 10mcg), and ofloxacin (oflox 5mcg)were employed. The performance of this test was checked by employing E. coli ATCC 25922 as a standard quality control organism.

Molecular typing

These isolates were subjected to detailed molecular typing. DNA isolation of 38 out of 40 isolates were done from pure growths from stool samples using standard techniques(Hi media bacterial DNA isolation kit) and PCR reactions to detect eaea and bfp gene using gene specific primers were done to categorize them as typical and atypical Enteropathogenic E. coli were done. The PCR procedure relies on detecting DNA sequences of interest amplified by a set of synthetic oligonucleotide primers.PCR products were then electrophoresed on agarose gel, stained with ethidium bromide and visualized by UV light.

Detection of virulence genes by PCR

For DNA amplification, G-Storm thermocycler was used. PCR amplified DNA fragments were separated by electrophoresis in agarose gel (1.4%) and stained with ethidium bromide (20 µg 100 ml^{-1}) at 3 V cm^{-1} for 3-4 hours. A sample without template DNA was included as a negative control in each experiment to check contamination. Electrophoretic profile was visualized under UV radiation and photographed with Geno Sens. Sizes of DNA fragments were estimated by comparison with standard Ladder 100 bp. Electrophoretic profiles were analyzed for polymorphism.

Detection of eaeA gene

A gradient PCR was done initially using Primers eae 1 and eae 2

- Reaction conditions were-

Initial denaturation	95˚c - 5min.	
Denaturation	95˚c – 30sec.	**40 cycle**
Annealing	49˚c to 55˚c	
Extension	72˚c – 75sec.	
Final extension	72˚c – 10min.	
Hold	4˚c - ∞	

Temperature gradients were as follows-

1- 49.1˚c
2- 49.2˚c
3- 49.5˚c
4- 50.1˚c
5- 50.8˚c
6- 51.6˚c
7- 52.3˚c
8- 53.2˚c
9- 54.1˚c
10- 54.6˚c
11- 54.9˚c
12- 55.1˚c

- Reaction mixture concentration and volume-

S.No.	Chemicals	Stock concentration	Final volume for 25ul
1.	DNA	25ng	2.5ul
2.	Taq buffer	10x	2.5ul
3.	$MgCl_2$	25mM	1.5ul
4.	dNTP	10mM	0.2ul
5.	P(F)	10pm	0.2ul
	P(R)	10pm	0.2ul
6.	Taq polymerase	5U	0.2ul
7.	ddw		17.7ul

Suitable temperature was found to be **55.1˚c** . The detection of eaea gene was done using primers described by Aranda et al,2006 (14).

PCR for bfpA gene detection

Gradient PCR using primers bfpAks & bfpAkcomas**.**

- Reaction conditions were-

Initial denaturation	95˚c - 5min.	
Denaturation	95˚c – 30sec.	**40 cycle**
Annealing	56˚c to 62˚c	
Extension	72˚c – 60sec.	
Final extension	72˚c – 10min.	
Hold	4˚c - ∞	

Temp. gradients are as follows-

1- 56.1˚c

2- 56.2˚c

3- 56.5˚c

4- 57.1˚c

5- 57.8˚c

6- 58.6˚c

7- 59.3˚c

8- 60.2˚c

9- 61.1˚c

10- 61.6˚c

11- 61.9˚c

12- 62.1˚c

❖ Reaction mixture concentration and volume-

S.No.	Chemicals	Stock concentration	Final volume for 25ul
1.	DNA	25ng	2.5ul
2.	Taq buffer	10x	2.5ul
3.	$MgCl_2$	25mM	1.5ul
4.	dNTP	10mM	0.2ul
5.	P(F)	10pm	0.2ul
	P(R)	10pm	0.2ul
6.	Taq polymerase	5U	0.2ul
7.	Ddw		17.7ul

Primers bfpAks and bfpAkcomas used were described by Lida et al 2006(15). Suitable temperature was found to be **56.1˚c** .

Results

The various pathogens detected in these 400 stool samples using stool microscopy, standard microbiological cultures for enterobacterieae, special stains for Candida and stool ELISA for cryptosporidium is shown in Table1. Some pathogen was isolated in a total of 98 out of 400 stool samples.

Table 1: Pathogens detected in 400 children with acute diarrhea.

Pathogen in stool	**Number (Percentage)**
Enteropathogenic E Coli.	40 (10%)
Enterotoxigenic E Coli	15(3.75%)
Enteroinvasive E Coli.	6 (1.5%)
Shigella spp	5(1.25%)
V.Cholerae spp	9(2.25%)
Candida spp	6(1.5%)
Entamoeba Histolytica	1(0.25%)
Cryptosporidium spp	16(4%)
No isolate	302

Out of the 400 stool samples cultured, 40 Enteropathogenic *E. coli* were detected after culture and serotyping using O specific antisera. Only those *E. coli* agglutinating with Polyvalent 1, Polyvalent 2 and Polyvalent 3 antisera, were classified as enteropathogenic as per the manual provided with the *E. coli* antisera kit. This was also in accordance with these serogroups being pathogenic as published earlier by Tamaki Y et al [13]. As per Tamaki Y et al, the likelihood of enteropathogenic *E. coli* in other polyvalent sera namely P4, P5, P6, P7 and P8 is very less likely.

The mean age of patients was12 months (range 2 months to 36 months) and the male female ratio was 1.85.Out of 40 children, 32(80%) had 6 to 24 stools per day. Vomiting was seen in 28 (70%) children. 31 children (77.5%) had fever at presentation. Dehydration was seen in 19(47.55%) cases. 28 children (70%) had anemia (hemoglobin less than 10 gm %). The other clinical features are summarized in Table 2. As regards nutritional status, 21 children (52.5%) were underweight or severely underweight. Stunting was seen in 11 (32.5%) cases. Some degree of wasting was seen in 17(42.5%) children (Table 3).

Antibiotic Sensitivity of EPEC Isolates

Antibiotic sensitivities, were tested towards antibiotics recommended by WHO namely Ciprofloxacin and Ceftriaxone. Other antibiotics freely available in the Indian market, and purchased by patients over the counter without prescriptions from a medical practitioner, were also tested. It was seen that the majority of isolates were resistant to commonly used antimicrobials (Table 4).

The resistance patterns to various antibiotics were as follows: Nalidixic acid 95%, Amoxicillin 90%, Cefotaxime 77.5%, Norfloxacin 77.5%, Ceftriaxone 75%, Ciprofloxacin 72.5%, Ofloxacin 70%, Nitrofurantoin 27.5%, Azithromycin 25%, Gentamicin 17.5%, and Amikacin 12.5%. The 40 enteropathogenic *E.coli* belonged to various serogroups, as shown in Table 4.

Table 2: Clinical and laboratory characteristics of 40 children with EPEC diarrhea.

Characteristics	Number (Percentages)
Frequency of loose stools	
< 6 per day	3 (7.5)
6-12 per day	18 (45)
12-24 per day	14 (35)
> 24 per day	5 (12.5)
Blood in stools	2 (5)
Presence of vomiting	28 (70)
Fever	
No fever	9 (22.5)
Low grade < 100ºF	4 (10)
Moderate grade 100-102ºF	10 (25)
High grade > 102º F	17 (42.5)
Degree of dehydration	
No dehydration	21 (52.5)
Some dehydration	17 (42.5)
Severe dehydration	2 (5)
Presence of metabolic acidosis on blood gas analysis	16 (40)
Anemia	
Hemoglobin <6 gm %	2 (5)
Hemoglobin 6-10 gm%	26 (65)
Hemoglobin > 10 gm %	12 (30)
Serum sodium	
Normal (135-150 meq/l)	31 (77.5)
Raised	2 (5)
Decreased	7 (17.5)
Serum potassium	
Normal (3.5 – 5 meq/l)	37 (92.5)
Raised	2 (5)
Decreased	1 (2.5)
Blood urea	
Normal	34 (85)
Raised	6 (15)

Table 3: Nutritional Status of 40 Children with EPEC Diarrhea

WEIGHT FOR AGE	**NORMAL WEIGHT N (%)** *19 (47.5%)*	**UNDERWEIGHT (-2 z score to -3z score) N (%)** *9 (22.5%)*	**SEVERELY UNDERWEIGHT (Less than -3 z score) N (%)** *12 (30)*
LENGTH/HEIGHT FOR AGE	**NORMAL LENGTH/HEIGHT N (%)** *27 (67.5%)*	**STUNTED (-2 z score to -3 z score) N (%)** *4 (10%)*	**SEVERELY STUNTED (Less than -3 z score) N (%)** *9 (22.5%)*
WEIGHT FOR LENGTH/ HEIGHT	**NORMAL WEIGHT FOR LENGTH/ HEIGHT N (%)** *23 (57.5%)*	**WASTED (-2 z score to -3 z score) N (%)** *4(10%)*	**SEVERELY WASTED (Less than -3 z score) N (%)** *13(32.5%)*

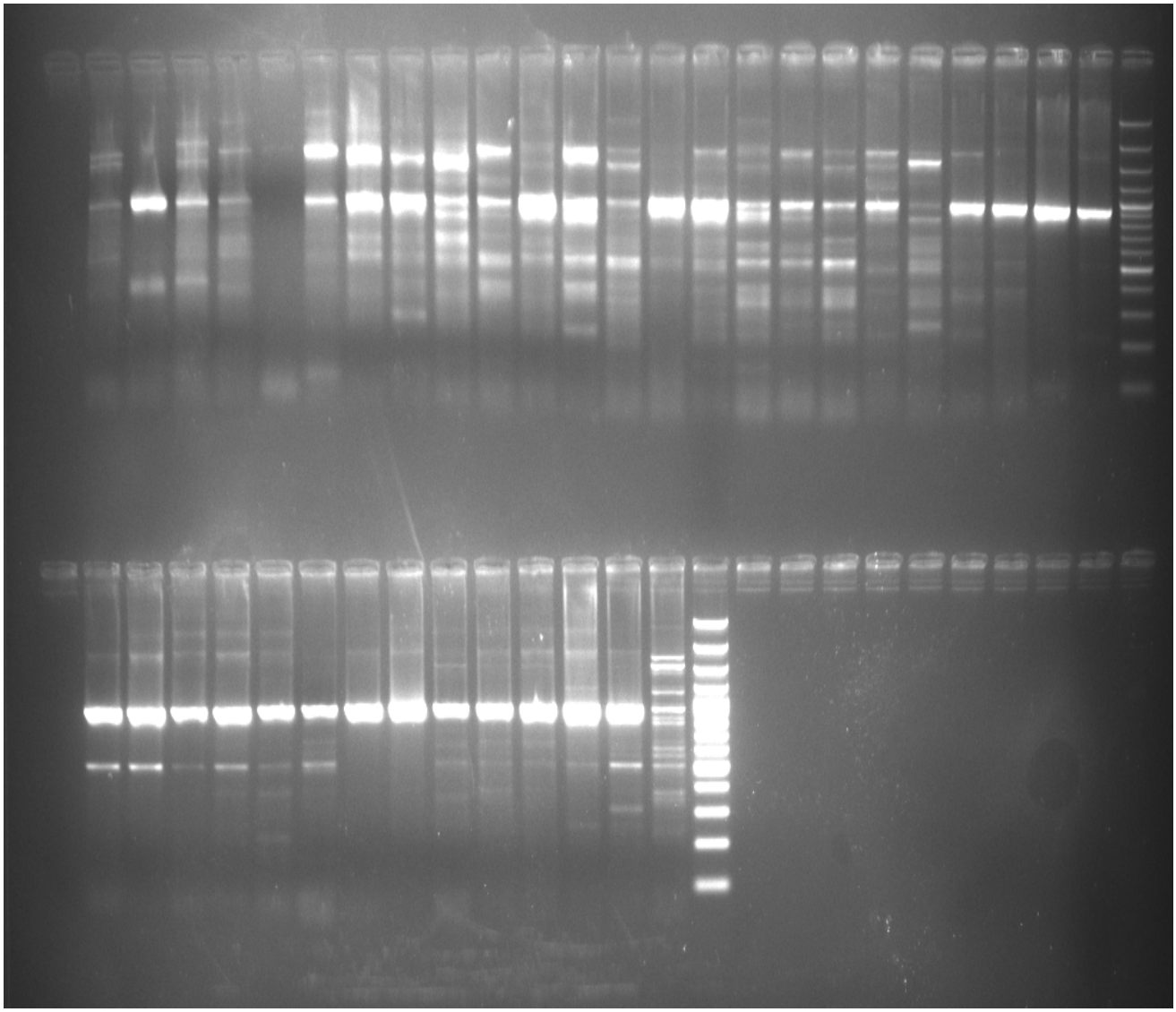
Table 4: Antibiotic Sensitivity patterns of 40 EPEC isolates from children with acute diarrhea

EPEC Serotype	Genta	Amika	Ceftriax	Norflox	Oflox	Cipro	Nitrofuran	Azithro	Cefotax	Amoxy	Nalidixic acid
O157	S	S	IS	R	R	R	R	R	R	R	R

Polyvalent 3	R	R	R	S	S	S	S	IS	R	R	R
Polyvalent 1	S	S	IS	S	S	S	S	S	R	R	R
O128	S	S	R	R	R	IS	S	S	R	R	R
O1	IS	S	R	R	IS	R	S	S	R	R	R
O18	S	S	R	R	R	R	S	R	R	R	R
Polyvalent 2	S	S	S	S	S	S	S	S	R	R	R
O1	R	R	R	R	R	R	S	S	R	R	R
O146	S	S	R	R	R	R	S	S	R	R	R
O119	S	S	S	S	S	S	S	IS	IS	R	R
O44	S	S	S	S	S	S	S	S	S	R	R
O142	R	S	R	R	R	R	R	R	R	R	R
O1	R	R	R	R	R	R	IS	R	R	IS	R
O11	S	S	R	R	R	R	IS	R	R	R	IS
Polyvalent 3	S	S	R	R	R	R	S	S	R	R	R
O1	S	S	S	R	R	R	S	IS	R	R	R
Polyvalent 2	IS	S	R	R	R	R	IS	R	S	R	R
O26	S	S	S	R	R	R	R	IS	S	R	R
O126	S	S	R	S	S	S	S	S	S	S	R
O126	S	S	R	R	R	R	IS	S	R	R	R
O125	S	S	R	R	IS	IS	R	S	R	R	R
O1	R	IS	R	R	R	R	IS	S	R	R	R

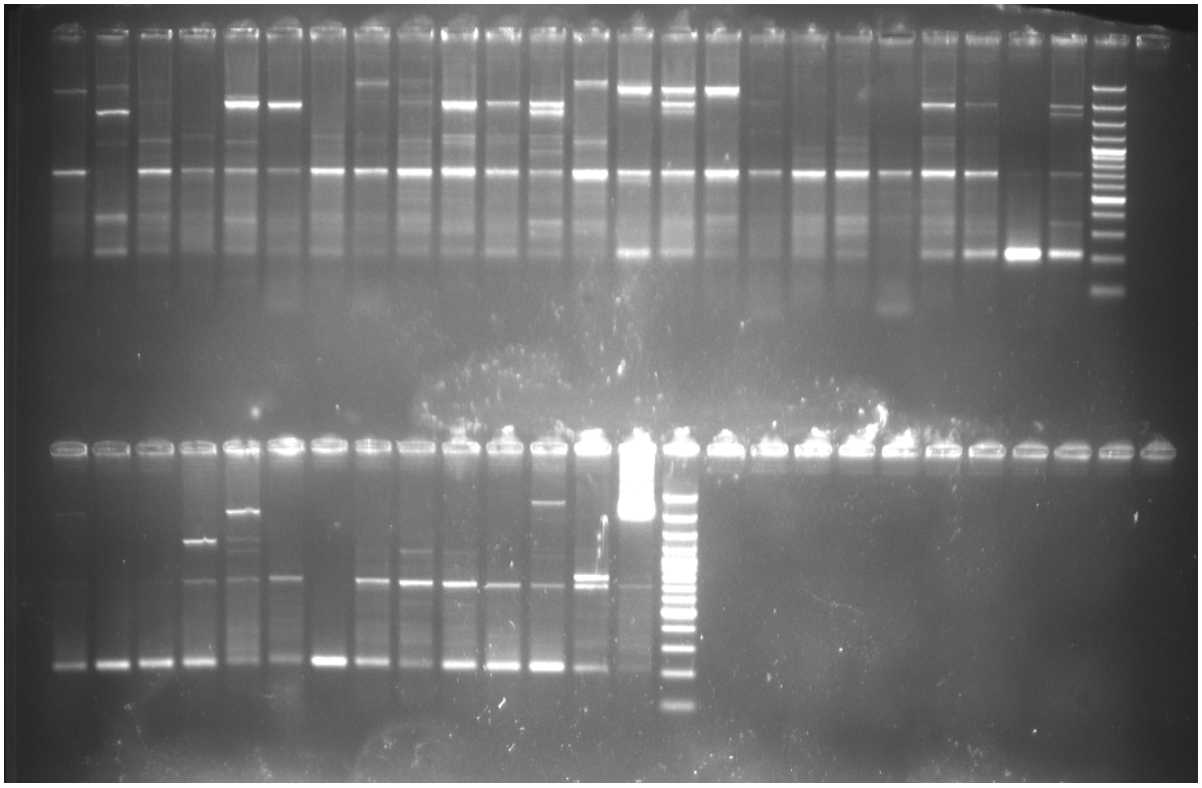
O1	S	S	R	IS	S	R	IS	S	R	R	R
Polyvalent 2	S	S	R	R	R	R	IS	S	R	R	R
O44	S	S	R	R	R	IS	R	S	R	IS	R
O114	S	S	R	R	R	R	R	S	R	R	R
O114	S	S	R	R	IS	R	R	R	IS	R	R
O146	S	S	S	S	S	S	S	S	S	S	R
O1	S	R	R	R	R	R	S	R	R	R	R
Polyvalent 2	R	S	R	R	R	R	R	S	R	R	R
O158	S	S	R	S	S	S	R	S	R	R	R
O114	S	R	R	R	R	R	S	S	IS	R	IS
O142	S	S	R	R	R	R	IS	S	IS	R	R
O18	R	S	R	R	R	R	R	IS	R	R	R
O166	S	S	S	R	R	R	S	S	R	R	R
O126	S	S	IS	R	R	R	IS	R	R	R	R
Polyvalent 2	S	S	R	R	R	R	S	S	R	R	R
O114	S	S	R	R	R	R	R	S	R	R	R
O86a	S	S	R	R	R	R	IS	S	R	R	R
Polyvalent 1	S	IS	R	R	R	R	IS	R	R	R	R

Fig. 1: Detection of eaea gene.



The detection of eaea gene was done as shown above. It was detected in all samples and was 1400 base pairs in size. Product size 900 base pairs amplified in all samples whereas 500 base pairs amplified in all except samples 6,7,9,11,14,15,23,24,31. 500 base pair band showed polymorphism.

Fig. 2: Detection of bfpA gene.



Detection of bfpA gene using primers of Lida et al revealed bfpA gene presence in all 38 samples indicating that all strains were typical EPEC strains.

Discussion

Acute diarrhea is of great concern, because of the considerable morbidity and mortality it causes worldwide. The causative bacteria, leading to acute diarrhea in Indian children requiring hospitalization, remains unclear. Recent literature highlighting various etiologies of diarrhea among hospitalized children is rare.

Although diarrheagenic *E.coli* pathotypes are well recognized, they are not routinely sought as enteric pathogens in clinical laboratories in India, owing to a lack of adequate infrastructure, like anti-sera and advanced molecular techniques, described extensively in medical literature. Thus, the exact burden of *E.coli* diarrhea, among hospitalized children across various tertiary level

medical institutes in India, is still far from clear. Medical literature on clinical spectrum of diarrhea, due to enteropathogenic *E.coli,* is non-existent. Most published articles, have focused only on some microbiological aspects of the organism, like molecular typing etc, with scant details on the clinical aspects of this important issue.

Recently, emergence of widespread antibiotic resistance is an important public health problem. At our institute, we have witnessed a lack of clinical response to standard first line antimicrobials like ciprofloxacin, recommended by WHO as first line antibacterial for diarrhea among children. Also, safe and effective antibiotics for acute diarrhea in children are very few. It is possible that widespread over-the-counter sale of these drugs has led to large scale antibiotic resistance. These clinical observations prompted us to carry out a systematic study on this important clinical problem.

The study was conducted to determine the clinical spectrum of acute diarrhea due to enteropathogenic *E.coli* (EPEC) and to study the antibiotic sensitivity patterns of these isolates. Among the 400 stool samples cultured, enteropathogenic *E coli* was detected in 40, amounting to a 10% incidence. In a systematic review of pediatric diarrhea etiology, using 266 studies published between 1990 and 2002, EPEC were still identified as being among the most important pathogens, with a median prevalence of 8.8% (IQR, inter-quartile range, 6.6–13.2) in the community setting, 9.1% (IQR 4.5–19.4) in the outpatient setting and 15.6 % (IQR 8.3–27.5) in the inpatient setting [7]. In this context, EPEC was the second most common cause of inpatient diarrhea after rotavirus (25.4%).

However, there are important regional and temporal variations. In a recent study of hospitalized diarrheal patients in India, EPEC was responsible for 3.2% of 648 diarrhea samples in children younger than 5 year of age [16]. In another study from Italy, pathogenic *E.coli* was seen in only 6.3% cases [17]. Our study, was similar to a publication from Vellore, South India, where the incidence of enteropathogenic *E.coli* was 9.9% [18]. The rate of isolation of the EPEC was much higher in Chile (38.3%) and Brazil (34.0%), whereas the frequency was lowest in Somalia (4.0%) and Thailand (5.5%) [19-21]. These publications, however, do not highlight any clinical aspects of this disease.

The clinical characteristics of EPEC diarrhea in Indian children have not been completely described, since only few studies have searched for all common pathogens. In this study, we found that children with EPEC diarrhea is characterized by the presence of fever, profuse watery stools, more than 6 stools per 24 hours, associated with vomiting and concomitant malnutrition, in a child less than 2 years of age, during the onset of summer. It was noticed that all these children were not exclusively breast fed till 6 months of age and were on diluted milk feeds with poor bottle hygiene. The lack of adequate complimentary feeding in these children coming from impoverished backgrounds, contributed to widespread malnutrition and a propensity for acquisition of diarrhea with the advent of summer. Also, a lack of awareness of correct handwashing techniques was widely prevalent. Inadequate access to safe drinking water, facilitated spread of these bacterial pathogens.

Differentiation between the diarrheagenic *E. coli* pathotypes is of great importance, since they are involved in acute diarrheal diseases and may require specific antimicrobial chemotherapy. In relation to treatment, few studies have evaluated in a systematic manner, the value of antimicrobials for the management of EPEC infection in children. The high antimicrobial resistance observed in our study, raises a broad discussion on the indiscriminate or improper use of antimicrobials. There are no studies highlighting the antibiotic resistance patterns of enteropathogenic *E.coli* diarrhea in children from India.

World Health Organization recommends use of ciprofloxacin as the first line drug, for management of diarrhea in children. The emergence of widespread resistance to quinolones, as seen in this study, and also to most commonly used injectable antimicrobials, like ceftriaxone and cefotaxime, is indeed alarming. Lack of resistance to aminoglycosides, namely gentamicin and amikacin, is noteworthy. Monotherapy with these drugs, may be useful and cheap in developing countries, like India. The advantages of aminoglycosides, include once a day administration using intramuscular route. This may prove extremely beneficial in smaller hospitals and other health centres treating pediatric diarrhea.

For diagnosis, polymerase chain reaction (PCR) techniques, should be used for the proper identification of EPEC. Molecular characterization of 38 isolates revealed that these strains were typical EPEC strains due to presence of eaea gene and bfp gene in all samples.Molecular methods are still not easily available in clinical laboratories in India as they are expensive. The

E. coli O specific antisera, are a rapid and easy test for laboratory screening for detection of EPEC, in areas where molecular typing for virulence genes are not routinely available.

This study highlights that, multidrug resistant enteropathogenic *E. coli*, can be associated with childhood diarrhea, in children from Northern India. Finally, this was a hospital based study of mostly severe diarrheal episodes, and these results may differ from less severe or community treated diarrheal cases. It is possible, that only severe forms of EPEC diarrhea, seen in children with concomitant malnutrition reported to this tertiary level hospital. In the community, diarrheal disease due to EPEC in well nourished children, might be a self-limiting illness. Diarrhea may result from an interplay of multiple factors in hospitalized children, including host susceptibility (child's age, absence of breastfeeding, poor nutritional and immunological status), and environmental factors (poor hygiene and high fecal contamination). Thus, it is possible that interplay of various factors, made the diarrhea episodes more severe, requiring hospitalization and antibiotic therapy.

References

[1]. Black RE, Cousens S, Johnson HL, Lawn JE, Rudan I, Bassani DG, et al. Global, regional, and national causes of child mortality in 2008: a systematic analysis. Lancet. 2010; 375:1969–87.

[2]. Bassani DG, Kumar R, Awasthi S, Morris SK, Paul VK et al.; Million Death Study Collaborators. Causes of neonatal and child mortality in India: a nationally representative mortality survey. Lancet 2010;376:1853-60. doi: 10.1016/S0140-6736(10)61461-4 .

[3]. Bryce J, Boschi-Pinto C, Shibuya K, Black RE. WHO estimates of the

causes of death in children. Lancet 2005; 365:1147–52.

[4]. FischerWalker CL, Friberg IK, Binkin N, et al. Scaling up diarrhea prevention

and treatment interventions: a Lives Saved Tool analysis. PLoS Medicine 2011; 8:e1000428.

[5]. O'Ryan M, Prado V, Pickering LK . A millennium update on pediatric

diarrheal illness in the developing world. Semin Pediatr Infect Dis 2005 16: 125–136.

[6]. Clarke SC. Diarrhoeagenic Escherichia coli-an emerging problem? Diagn Microbiol Infect Dis 2001 41: 93–98.

[7]. Lanata, CF.; Walter, M.; BR, E. [Accessed 20 May 2014] Improving diarrhoea estimates. . WHO 2002. 2002.

http://www.who.int/child_adolescent_health/documents/pdfs/improving_diarrhoea _estimates.pdf .

[8]. Abba K, Sinfield R, Hart CA, Garner P. Pathogens associated with persistent diarrhoea in children in low and middle income countries: systematic review. BMC Infect Dis. 2009; 9:88.

[9]. Diarrhoea: Why children are still dying and what can be done. Geneva, WHO/UNICEF, 2009.

[10]. 'The Treatment of Diarrhoea – A manual for physicians and other senior health workers', WHO/CAH/03.7, World Health Organization, Geneva.

http://whqlibdoc.who.int/publications/2005/9241593180.pdf

[11]. Guidelines on Standard Operating Procedures for Microbiology. World Health Organization May 2000.

[12]. Tamaki Y, Narimatsu H, Miyazato T,Nakasone N,Higa N,Toma C,IwanagaM. Jpn J Infect Dis 2005:58:65-69.

[13]. Clinical and Laboratory Standards Institute, "Performance standards for antimicrobial susceptibility testing document M100-S19, CLSI, Wayne," pp. 52, 2009

[14]. Aranda KRS, Faeundes –Neto U , Scaletsky ICA .Evaluation of multiplex PCR for diagnosis of infection with diarreagenic Escherichia coli and Shigella spp . J Clin Microbio 2004 ;42(12):5849-5853.

[15]. Lida M, Yamazaki M, Yatsuyanagi J, Ratchtrachenchai OA,Subpasu S,Okamura N, Ito K.. Microbiol. Immunol. 2006; 50(9), 713–717.

[16].Nair GB, Ramamurthy T, Bhattacharya MK, Krishnan T, Ganguly S, Saha DR, et al. Emerging trends in the etiology of enteric pathogens as evidenced from an active surveillance of hospitalized diarrhoeal patients in Kolkata, India. Gut Pathog. 2010; 2:4.

[17]. Amisano G, Fornasero S, Migliaretti G, Caramello S, Tarasco V, Savino F. New Microbiologica 2011; 34**:** 45-51.

[18].Rajendran P, Ajjampur SSR, Chidambaram D et al. Diagn Microbiol Infect Dis2010 October ; 68(2): 117–122.

[19].Casalino M, Yusuf MW, Nicoletti M, Bazzicalupo P, Coppo A, et al. A two-year study of enteric infections associated with diarrhoeal diseases in children in urban Somalia. Trans R Soc Trop Med Hyg 1988;82: 637–641.

[20].Echeverria P, Orskov F, Orskov I, Knutton S, Scheutz F, et al. Attaching and effacing enteropathogenic Escherichia coli as a cause of infantile diarrhea in Bangkok. J Infect Dis 1991;164: 550–554.

[21].Chen HD, Frankel G . Enteropathogenic Escherichia coli: unravelling pathogenesis. FEMS Microbiol Rev2005; 29: 83–98.