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ISOLATION AND DETECTION OF ANTIMICROBIAL SUSCEPTIBILITY PATTERN AND ESBL PRODUCTION OF ESCHERICHIA COLI IN CHILDREN WITH GASTROENTERITIS

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ABSTRACT

Diarrhoeagenic *E.coli* is the most common bacterial pathogen causing childhood diarrhoea. In this study 100 stool samples from children having diarrhoea and 25 samples from healthy children were collected and 39 and 7 *E.coli* was isolated respectively. The strains were screened for ESBL production. 28 isolate (71.8%) in double disc synergy method and 22 isolate (56.4%) in phenotypic confirmation method showed ESBL production. In phenotypic method by using PCR, 24 (61.5%) isolate showed CTX-M gene and 3 (7.7%) isolate showed SHV gene. This study showed high prevalence of ESBL in *E.coli* strains which warrants effective screening technique in routine laboratories and usage of combination treatment regimen with Clavulanic acid to overcome the treatment failure.

KEYWORDS: ***Escherchia coli*, ESBL, Clavulanic acid, double disc synergy test, PCR.**

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INTRODUCTION

Diarrhoea is one of the scourges of mankind, killing millions of children all over the world especially in the developing countries. Diarrhoeal disease is an important public health problem with an estimated 750 million to 1 billion episodes of acute diarrhoeal illness occurring in children less than 3 years of age. When WHO initiated the diarrhoeal disease control program in 1980 approximately there were 744 to 1000 million episodes of diarrhoea and 4 to 6 million deaths every year[1]. *E.coli* is the major pathogen causing infantile diarrhoea less than five years of age. Most of the reports from the globe reveal high prevalence of *E.coli* causing infantile diarrhoea next only to Rotavirus. The increased prevalence of bacterial pathogens producing extended spectrum β-lactamases creates a requirement for laboratory testing methods that can accurately detect the presence of these enzymes in clinical isolates[2]. The concern for accurate detection of the extended spectrum β-lactamase is three fold, first being a worldwide problem of increasing extended spectrum β-lactamases prevalence, second is strain producing the extended spectrum β-lactamases are at high risk of resulting in treatment failure in critical clinical emergency, thirdly to find out the occurrence of those in individual clinical setups[3]. An extended spectrum β-lactamase specifically inhibited by β-lactamase inhibitor like Clavulanic acid and this property is utilized for the detection and confirmation of extended spectrum β-lactamases[4].

MATERIALS AND METHODS

The study group consisted of 100 children of less than 5 years of age with acute watery diarrhoea, 24 to 48 hours of duration with or without blood, and no intake of antibiotics for the past 2 weeks.25 healthy children with no history of diarrhoea for 2 weeks and matched for the age group, gender were included for comparison with the study group. All stool samples collected from study as well as control group were processed and pathogens were identified using standard microbiological procedures.

ANTIBIOTIC SUSCEPTIBILITY TEST:

Antimicrobial susceptibility of the organism was tested by Kirby Bauer Disc diffusion method recommended by Clinical Laboratory Standard Institute (CLSI) with Ampicillin 30mcg, Co-trimoxazole 23.75 mcg, Norfloxacin 10 mcg, Nalidixic acid 30 mcg, Gentamycin 10 mcg, Amikacin 30 mcg, Ofloxacin 5 mcg, Cefotaxime 30 mcg, Ceftazidime 30 mcg discs. *E.coli* ATCC 25992 was used as a quality control reference strain for testing gram negative enteric organisms.

DETECTION OF ESBL: Phenotypic method:

1. Double disc synergy test:

Isolates were grown to 0.5 Mc Farland's standard in Nutrient broth and lawn culture of it was made on a Muller Hinton agar plate. Discs of 3rd generation cephalosporin (cefotaxime and ceftazidime) were placed 20mm apart from an amoxicillin and clavulanic acid combined disc centre to centre. If inhibition around the 3rd generation cephalosporins showed a clear extension towards clavulanic acid disc, it was considered as ESBL producer.

2. Phenotypic confirmation disc diffusion test: Lawn culture of the organism was made and 3rd generation cephalosporin, ceftazidime disc (30mcg) was tested alone and along with their combination of clavulanic acid (10mcg).Organisms with 5mm increase in zone of inhibition for ceftazidime/ clavulanic acid (30/10mcg) combined discs are confirmed as ESBL producer.

GENOTYPIC METHOD:

POLYMERASE CHAIN REACTION

Extraction of DNA:

DNA was isolated from the positive cultures using UNIFLEX DNA ISOLATION KIT supplied by Bangalore Genei (India) Pvt. Ltd as per the instructions given by manufacturer.

PCR AMPLIFICATION:

Taq DNA polymerase supplied by Bangalore Genei (India) Pvt. Ltd was used. A PCR amplification assay was carried out using 10 pmol of each universal degenerated primer CTX M-F: 5'-AAA AAT CAC TGC GCC AGT TC-3'and CTX M-R: 5'-AGC TTA TTC ATC GCC ACG TT-3' targeting the CTX-M and SHV-F: 5'-TCC ACC ATC CAC TGC AGC AGC T-3' and SHV-R: 5'-AAC GGA ACT GAA TGA GGC GCT-3' targeting the SHV genes.PCR amplification conditions were as follows: initial denaturation step at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s; annealing at 55°C for 1 min 20 s; extension at 72°C for 1 min 20 sec, and a final extension step at 72°C for 5 min. Subsequently, PCR-amplicons were separated electrophoretically on a precast 1.5% agarose gel.

RESULTS

One hundred children below the age groups of 5 years were screened for *E.coli* and enteric pathogens. 41 (41%) children were in the age group of 0-12 months and this was followed by 23 (23%) children in the age group of 13-24 months. Of the 25 control subjects screened, 9(36%) were in the age group of 0-12 months and 5(20%) were in the age group of 13-24 months (Table: 1).Among the one hundred diarrhoeal children 60% were male and 40% were female. In the 25 control subjects, 64% were male and 36% were female (Table: 2).

Table 1

Age distribution of 100children with diarrhea and 25 control children

Age (months)	Diarrhoeal children n = 100		Control children n = 25	
	No.	%	No.	%
0-12	41	41	9	36
13-24	23	23	5	20
25-36	11	11	4	16
37-48	9	9	3	12
49-60	16	16	4	16

Table 2

Sex distribution of diarrhoeal and control children

SEX	Diarrhoeal children n = 100		Control children n = 25	
	No.	%	No.	%
Male	60	60	16	64
Female	40	40	9	36

Out of 100 samples screened for bacterial pathogens, the predominant isolate was *E.coli* in 39% followed by, *Salmonella* spp in 1%. In the control group, *E.coli* was isolated from 7(28%) stool specimens. (Table: 3).

Table 3
Bacterial enteric pathogens isolated from study and control groups

Bacterial pathogen isolated	Diarrhoeal children n = 100		Control children n = 25	
	No.	Percentage	No.	Percentage
E.coli	39	39	7	28
Salmonella spp	1	1	_	_

The antibiotic resistant pattern of *E.coli* in our study group was found to be 94.9% for Ampicillin, followed by 79.5% for both Cotrimoxazole and Nalidixic acid and 59% for Norfloxacin. Of the cephalosporins 43.6% were resistant for both Cefotaxime and Ceftazidime. Thirty four (87.2%) strains were susceptible to Amikacin followed by 23 strains (59%) for Gentamycin. (Table: 4). In control group all (100%) 7 strains were resistant to Ampicillin and 6 (85.7%) strains were sensitive to Amikacin. Three (42.8%) strains were sensitive to for both Cefotaxime and Ceftazidime (Table: 5).

Table 4
Antimicrobial susceptibility pattern of E.coli isolates in study group (n = 39)

S. no.	Antibiotics	Sensitive		Moderately sensitive		Resistant	
		No.	%	No.	%	No.	%
1.	Ampicillin(30mcg)	0	0	2	5.1	37	94.9
2.	Cotrimoxazole(23.75mcg)	6	15.4	2	5.1	31	79.5
3.	Amikacin(30mcg)	34	87.2	2	5.1	3	7.7
4.	Gentamycin(10mcg)	23	59	7	18	9	23
5.	Cefotaxime(30mcg)	14	35.9	8	20.5	17	43.6
6.	Ceftazidime(30mcg)	8	20.5	14	35.9	17	43.6
7.	Ofloxacin(5mcg)	15	38.5	9	23	15	38.5
8.	Norfloxacin(10mcg)	15	38.5	1	2.5	23	59
9.	Nalidixicacid(30mcg)	6	15.4	2	5.1	31	79.5

Table 5
Antimicrobial susceptibility pattern of E.coli isolates in control group (n = 7)

S.no.	Antibiotics	Sensitive		Moderately sensitive		Resistant	
		No.	%	No.	%	No.	%
1.	Ampicillin(30mcg)	0	0	0	0	7	100%
2.	Cotrimoxazole(23.75mcg)	1	14.3	1	14.3	5	71.4
3.	Amikacin(30mcg)	6	85.7	1	14.3	0	0
4.	Gentamycin(10mcg)	4	57.1	1	14.3	2	28.6
5.	Cefotaxime(30mcg)	3	42.8	2	28.6	2	28.6
6.	Ceftazidime(30mcg)	3	42.8	1	14.3	3	42.8
7.	Ofloxacin(5mcg)	4	57.1	2	28.6	1	14.3
8.	Norfloxacin(10mcg)	3	42.8	3	42.8	1	14.3
9.	Nalidixicacid(30mcg)	2	28.6	2	28.6	3	42.8

Of the 39 *E.coli* strains subjected to screening test for ESBL by double disc synergy test, 28 (71.8%) isolates produced extended zone of inhibition towards clavulanic acid disc. In the control groups 4(57.2%) isolates were positive for ESBL production. (Table: 6).In phenotypic confirmation disc diffusion test for confirmation of ESBL, 22(56.4%) of test isolates were positive for ESBL. In control isolates 3(42.8%) strains were positive. (Table: 7).

Table 6
Results of double disc synergy test for detection of ESBL

ESBL	Diarrhoeal children n = 39		Control children n = 7	
	No.	Percentage	No.	Percentage
POSITIVE	28	71.8	4	57.2
NEGATIVE	11	28.2	3	42.8

Figure 1
Phenotypic confirmation disc diffusion test (PCDDT) showing ESBL production

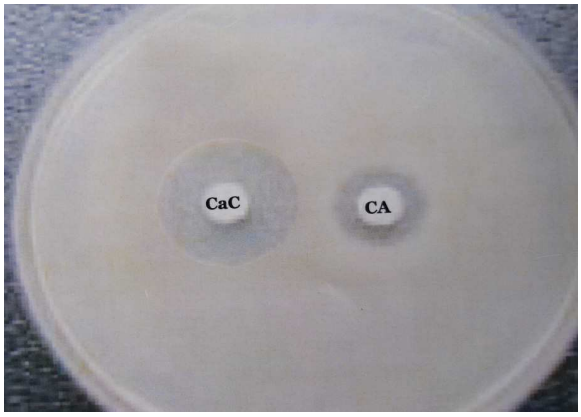


Table 7
Results of phenotypic confirmation disc diffusion test

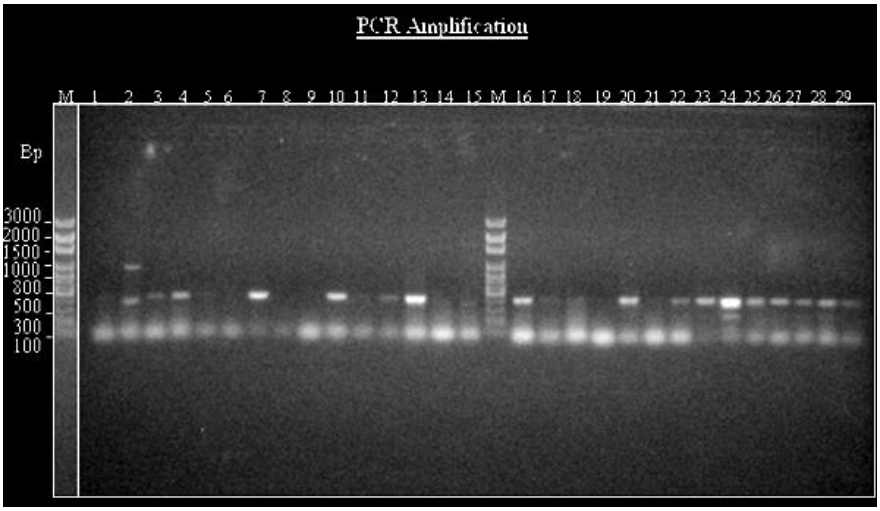
ESBL	Diarrhoeal children n = 39		Control children n = 7	
	No.	Percentage	No.	Percentage
POSITIVE	22	56.4	3	42.8
NEGATIVE	17	43.6	4	57.2

In genotypic ESBL detection by using PCR technique among the 39 test isolates, 24 isolates (61.5%) were positive for CTX-M gene and 3 isolates (7.7%) were positive for SHV gene. Among the 7 control strains 4 isolates (57.2%) were positive for CTX-M gene and all were negative for SHV gene (Table: 8).

Table 8
Results of genotypic ESBL detection test (PCR)

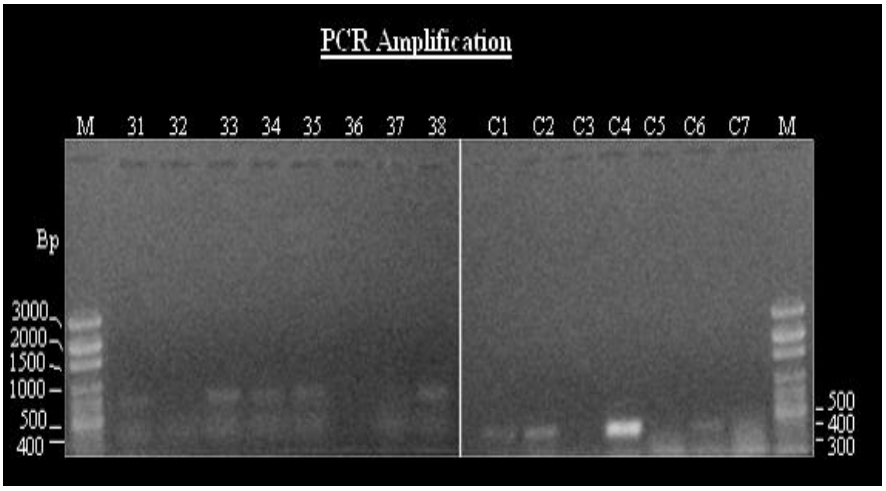
ESBL GENE	Diarrhoeal children n = 39		Control children n = 7	
	No.	Percentage	No.	Percentage
CTX-M	24	61.5	4	57.2
SHV	3	7.7	0	0

Figure 2
Agarose Gel Electrophoresis showing amplified ESBL genes (CTX-M)



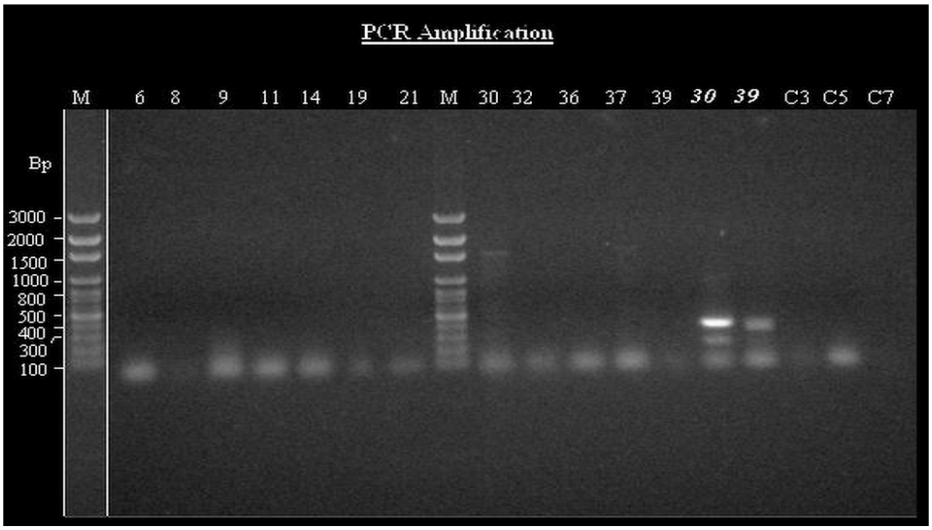
1-29 = Test isolates

Figure 3
Agarose Gel Electrophoresis showing amplified ESBL genes (CTX-M)



31-38 = Test isolates; C1-C7= Control isolates

Figure 4
Agarose Gel Electrophoresis showing amplified ESBL genes (SHV)



6,8,9,11,14,19,21,30,32,36,37,39= Test isolates; C3,C5,C7= Control isolates

DISCUSSION

Diarrhoeal diseases represent a major health problem in developing countries and also a high risk to travellers who visit these countries. Conservative estimates place the global death toll from diarrhoeal diseases at about two million deaths per year (1.7 - 2.5 million deaths), ranking third among all causes of infectious disease deaths worldwide. Most of these deaths occur in children under five years of age [5]. In many studies, pathogens are identified in atleast about 50- 60% of stool samples from children with acute diarrhoea. Among them diarrhoeagenic *E.coli* and rotavirus are the most common[6]. Torres et al[7] in his study observed that *E.coli* was the most frequent bacterial isolate identified (39.3%) in Uruguay from diarrhoeal children. Sabrina Moyo et al[8] in Tanzania isolated 64 (22.9%) *E.coli* strains from 280 diarrhoeal children under five years of age. Samuel Vilchez et al[9] from Nicaragua observed 53.8% prevalence of *E.coli* in diarrhoeal children and 53.1% prevalence in control group. John Albert et al[10] in his study in Bangladesh isolated *E.coli* (45.2%), *Salmonella* spp.(1.1%), *Shigella* spp. (10.2%), Vibrio (9.5%) and Rotavirus (16.9%) from the stool samples of children under 5 years of age. Khundkar Hasan et al[11] in Bangladesh noted 46.78% *E.coli* prevalence in diarrhoea children and 36.6% prevalence in control group.

In our study we observed *E.coli* was the major bacterial pathogen isolated from stool samples of diarrhoeal children less than five years of age. We have isolated 39 *E.coli* strains from 100 samples and 1 strain of salmonella spp. This correlates with the study conducted by Torres et al. In the control group we isolated 7 (28%) *E.coli* strains among the 25 control subjects. The prevalence of *E.coli* varied worldwide and it was 2.2% in a study carried out in Vietnam [12], 10% in children from Djibouti [13] and 17% in Bangladeshi children[14]. Farid Abu-Elamreen et al[15] in their study observed high incidence of diarrhoea(63.3%) in the first year of life. In our present study, a higher incidence of diarrhoea that is 41% occurred in children below one year of age and 23% in the second year of life. Jafari et al[16] in Iran reported that the highest incidence were found in children less than 1-year-old (42.7%). Sabrino Mayo et al[8] in a study observed that incidence of diarrhoea in males (61.4%) was higher than female children. Similar observation was made by Shanta Dutta et al[17] in 2001 with male prevalence of 61%. Farid

Abu-Elamreen et al[15] in his study in Palestine showed higher incidence of diarrhoea in males (59%). Our study correlates with other studies in which we have observed that the incidence of diarrhoea in children was 60% in males and 40% in females. Torres et al[7] reported 96.2% of *E.coli* was resistant to Ampicillin in their study. They also noted the strains were 40% and 16.2% resistant to Co-trimoxazole and Gentamycin respectively. Rupal Mody et al[18] in USA reported that *E.coli* was 100% resistant to ampicillin, 97% sensitive to amikacin, 45% sensitive to gentamycin, 97% resistant to ceftrioxazone and 97% resistant to ceftazidime. Patel et al[19] in Gujarat noted that *E.coli* was 69.23% sensitive to amikacin in their study In our present study we have noted that 94.9% of *E.coli* was resistant to Ampicillin which correlates with most of the other studies and 79.5% were resistant to co-trimoxazole. In our study *E.coli* showed 87.2% sensitivity with amikacin and 59% sensitivity with Gentmycin and 79.5% of strains were resistant to Nalidixic acid. Resistance to cefotaxime and ceftazidime was noted as 43.6% for both. This was slightly lesser than other studies in India.

Menon et al[4] in Chennai identified that 20% of their isolates were ESBL positive in double disc synergy method. Further, some studies[20] showed ESBL prevalence between 6.6% to 68% in India. Jain et al[21] reported that 63.6% of *E.coli* strains were ESBL producers. In Tamilnadu Babypadmini et al[22] in their study showed 41% ESBL positivity among *E.coli* strains. In our present study we have performed double disc synergy method to screen for ESBL producer in both test and control strains. We have noted 71.8% ESBL positive strains in diarrhoeal group and 57.2% positive in control group.

A study by Rodrigues in Maharashtra[23] reported 65.8% of ESBL positivity in phenotypic methods. In our study in phenotypic confirmation disc method we have found 56.4% ESBL positivity in diarrhoeal group and 42.8% positivity in control groups. This was lesser than the screening test result. Melissa McCracken[24] in North America noted that disc diffusion confirmation test with cefotaxime alone and in combination with clavulanic acid was 100% sensitive in detecting CTX-M type of ESBL producer.

Babypadmini et al[22] in Tamilnadu noted that among the 23 isolates 19 isolates (82.6%) revealed the presence of CTX-M type of ESBL gene. A study conducted in Spain[25] showed a predominance of CTX-M type (66.2%) and SHV type (20.6%). Munday et al[26] in UK noted that ESBL producing isolates were found in faeces samples of both the community and patients in hospitals, with the CTX-M type being the most common. Johann Pitout et al[27] found in 73% of the ESBL-producing *E. coli* isolates were positive for CTX-M genes and 15% were SHV positive. In our study we have observed that 61.5% of isolates showed CTX-M type of ESBL and 7.7% of isolates showed SHV type of ESBL gene from the isolates of diarrhoeal children. In control group 57.2% of isolates showed CTX-M type of ESBL and there was no SHV type of ESBL gene. Like other studies in India we have also noted that CTX-M type was the predominant ESBL type in *E.coli* from faeces samples of children with acute gastroenteritis. A study conducted by Melissa McCracken[24] in North America noted that SHV types of ESBL were frequently seen among Salmonella spp. whereas CTX-M type of ESBL were prevalent more among *E.coli* isolates. This may explain the high incidence of CTX-M type of ESBL in our study.

CONCLUSION

Diarrhoeal incidence was common in children under one year of age and *E.coli* was the predominant isolate from stool samples of diarrhoeal and control children aged 0-5 years. Majority of the *E.coli* strains were resistant to many antibiotics. Since the prevalence of ESBL in *E.coli* was high, screening for ESBL is recommended in routine diagnostic laboratories. CTX-M was the most common ESBL producing gene present in *E.coli* strains in our setup. Use of Clavulanic acid in

combination with third generation cephalosporin might be a better option in the management of infections of ESBL producing *E.coli*.

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