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Research Article

Emergence of Extended Spectrum Beta Lactamases Producing Multi Drug Resistant Diarrheagenic *Escherichia coli* in Children Under Five Years

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Abstract

Extended-spectrum b-lactamase producing Diarrhoeagenic Escherichia coli (DEC) have spread rapidly worldwide and impose a serious threat to human health, especially in children. The aim of our study was to compare phenotypic and genotypic methods for the detection of Extended-Spectrum Beta-Lactamases in children less than five years of age. A total of 120 Diarrhoeagenic E.coli isolates were subjected to antibiotic susceptibility testing by disc-diffusion method as per CLSI guidelines followed by combined disc test for confirmation. Further, molecular identification of ESBL genes were performed by multiplex PCR. All isolates were examined for the presence of CTX-M, TEM, OXA and SHV genes. Among them, 79/120 (65.83%) isolates were resistant to cefotaxime and Ceftazidime, of which 76/120 (63.33%) were confirmed as ESBL positive by combined disc test. However, the numbers of isolates determined positive for ESBL by genotypic method were 77/120 (64.16%). One isolate showed the presence of all four ESBL genes. Eight out of 120 isolates (6.66%) were ESBL positive by PCR but negative by combined disc test. The results showed that some antibiotic sensitive isolates were also carrying ESBL genes. Such isolates have the potential to turn resistant later. The genotypic method for the detection of resistance genes was more consistent in comparison to the phenotypic methods.

Keywords: Extended Spectrum Beta Lactamases; diarrhoeagenic Escherichia coli; multiplex PCR

Introduction

One of the important resistant mechanisms in gram-negative bacteria against beta-lactam antibiotics is the production of beta-lac-tamase enzyme [1]. Evolution of beta lactamase enzyme was due to the use of new broad-spectrum antibiotics such as Cephalosporins which are used in the treatment of bacterial infections [2] Extended-spectrum Beta-lactamases (ESBLs) were first recognized in 1980s, in Klebsiella species and later in *Escherichia coli* and are currently spreading rapidly amongst other members of enterobacteriaceae, other gram-negative bacilli are currently spreading rapidly amongst other members of enterobacteriaceae, largely due to genes located on plasmids that can distribute across species barriers [3,4]. Infections with ESBL-producing bacteria are associated with nearly twice the mortality compared to non-ESBL producers [5] Carriage of resistance by commensal en-terobacteriaceae strains in the gut may serve as a reservoir of resistance genes that may subsequently be acquired by other strains that cause clinically significant infection [3]. More than 200 types of ESBLs have been found worldwide, which comprised of mainly 157 TEM, 101 SHV and 65 CTX-M variants [6,7]. The ESBL producing diarrhoeagenic E.coli can be detected either by phenotypic or genotypic methods. Phenotypic methods are not always dependable [8], hence genotypic methods have proven to be a more useful option for ESBL detection. More-over, carriage of ESBL producing diarrhoeagenic E.coli isolated from fecal samples in pediatric population can create a larger threat to the community through widespread transmission of these strains. Thus, the aim of our study was to detect the burden of ESBL producing diarrhoeagenic E.coli in fecal sample of children with diarrhea, children admitted to the hospital and on antibiotics for illness other than diarrhea and amongst healthy children, by phenotypic and genotypic methods.

Materials and Methods

Patients: The study population included three groups. Group 1 comprised of 40 children with acute diarrhea (less than 72 hour duration) attending the Out Patient Department (OPD) and not receiving any antibiotic. Group-2 comprised of 40 children hospitalized and receiv-ing antibiotic (oral or I/v for 72 hr or more or for conditions other than diarrhea). Group 3 included 40 healthy children below 5 years of age who were not suffering from diarrhea or any other disease, as controls. The study protocol was approved by institutional ethical committee.

Processing of samples

Isolation: Fresh stool samples were inoculated on MacConkey agar plates and incubated aerobically at 37°C for 24 hours and lactose fermenting colonies were identified as *E.coli* by standard biochemical tests [9]. Two or three lactose fermenting colonies, were selected and inoculated (lawn culture) on Muller Hinton agar for antibiotic susceptibility testing and DNA extraction. Diarrhoeagenic *E.coli* was detected by multiplex PCR for virulent genes [10].

Antimicrobial Susceptibility Testing

Antimicrobial Susceptibility Testing was performed on Mueller-Hinton agar plates by the Kirby Bauer disc diffusion method as per CLSI guidelines [11]. All clinical isolates were screened for ESBL production by phenotypic method. Zone of inhibition < 22 mm with Ceftazidime (30 µg) and ≤ 27 mm with Cefotaxime (30 µg) were considered as a potential ESBL producer and were further confirmed by combination disc diffusion test [12]. *E.coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative control and positive control respectively.

ESBL confirmation

Double Disk Synergy Test (DDST) was performed on ceftazidime/cefotaxime resistant strains by placing disks of Ceftazidime (30 µg) or cefotaxime (30 µg) at a distance of 20 mm from the ceftazidime/clavunate (20/10 µg) disc on Mueller-Hinton agar plates. Enhanced zone of inhibition ≥ 5 mm with ceftazidine/clavunate disc was considered as positive for ESBL production [12]. All diarrhoeagenic E.coli isolates irrespective of ESBL production by DDST were also tested for the presence of ESBL genes by multiplex PCR.

Primers used for amplifying the sequences were based on previously published literature [13,14]. Primer sequences were TEM F-AGT-GCTGCCATAACCATGAGG & R-CTGACTCCCCGTCGTGTAGATA (431bp), SHV-F-GATGAACGCTTTCCCATGATG & R-CGCTGTTATCGCTCATGG-TAA (214bp), OXA- F-ATTATCTACAGCAGCGCCAGTG & R-TGCATCCACGTCTTTGGTG (296bp), CTX-M- F-GACAAAGAGAGTGCAACGGATG & R-TCAGTGCGATCCAGACGAAA (501bp) and 16s RNA- F-CCCCCTGGACGAAGACTGAC & R-ACCGCTGGCAACAAAGGATA (401bp).

Genotypic Assay

DNA extraction: Lactose fermenting colonies on MacConkey agar were isolated and confirmed as *E.coli* biochemically and DEC were detected by multiplex PCR. Genomic DNA extraction was done using RBC (Real Biotech Corporation, Taiwan) kit as per manufacturer's instructions. All confirmed ESBL producing isolates by phenotypic method were subjected to molecular testing to detect ESBL producing genes.

PCR conditions: Each multiplex PCR test was performed in 0.2 ml PCR tube, containing a total volume of 25 µl including, 2.5 µl buffer (10X), 1 µl dNTP's (200 µM), 1.5 µl MgCL2 (1.5 mM), 1 µL each primer (10 µM for each primer forward and reverse), 0.25 ul of Taq DNA Polymerase (1000 unit), 5ul of the DNA and water to make up the volume. All the four primer pairs of ESBL genes were added in the same tube along with 16sRNA gene primer [14]. All PCR reagents were purchased from New England Biolabs (NEB, UK) and amplification was performed on a thermo cycler (Eppendorf, Germany).

PCR cycle: PCR tubes were subjected to an initial denaturation of 95°C for 10 minutes followed by 35 amplification cycles of 40 sec at 94°C, 30 sec at 60°C and 40 sec at 72°C, final extension at 72°C for 7 minutes and cooling at 4°C [15]. Amplified PCR products were analyzed on 1% agarose gel stained with ethidium bromide by gel electrophoresis at 140 volts for 25 minutes in a 10 well apparatus. A molecular marker of 100bp was used to determine the size of the amplicons (Genei, Bangalore). 16s RNA (401bp) was also used as internal quality control [16].

DNA sequencing was carried in some of the positive ESBL producing PCR products for confirmation of results with the same set of primers as mentioned above. Purification and DNA sequencing of the PCR products was done commercially (Yaazh Xenomics, Chennai). Basic Local Alignment Search Tool (BLAST) was used for initial sequence analysis (http://www.ncbi.nlm.nih.gov/blast). Clustal W_2 & MEGA (Molecular Evolutionary Genetics Analysis) software version 6.06 were used for further analysis and phylogenetic tree production.

Statistical Analysis: Statistical Analysis was done using Statistical Package for Social Sciences (SPSS) (SPSS; Version 20.0). Chi square test and Fisher's exact test were used to determine statistical significance of data. P value < 0.05 was considered significant. Multivariable logistic regression was done to determine the risk of predominant ESBL infection with increase in age in the three groups, of study subjects.

Results

Total of 120 stool specimens, over a period of one year from July 2012 to July 2013 were collected from children from different categories as mentioned earlier. There was a male preponderance of 60.83% versus 39.16% of females with a mean age of 1.91 years (P> 0.05) in all the groups. DEC was detected in 106/120 (88.33%) isolates that included 39, 40 and 27 isolates in three groups respectively as detected by PCR (data not shown).

The antibiotic resistance pattern of DEC isolates is depicted in Table 1. The drug susceptibility pattern of rest of non diarrhgenic *E.coli* was similar as that of DEC.

A comparative distribution of ESBL production by phenotypic and genotypic methods is depicted in Table 2.

An effort was made to explore the association of ESBL infection with two age groups in Table 3. The odds of presence of ESBL infection in group 1 was 2.455 times higher when compared to the odds of presence of ESBL infection in group 3.Similarly, the odds of presence of ESBL infection in group 2 was 1.909 times higher in comparison to the odds of the presence of ESBL infection in group 3. However; P value was not found to be significant in group 1 and 2 when compared with group 3.

Antibiotic resistant	Group 1 n=39 %)	Group 2 n=40 (%)	Group 3 n=27 (%)	Total n=106(%)	P value
Norfloxacin (NOR)	9 (23.07)	7 (17.5)	10 (37.03)	26 (24.52)	0.709
Cefotaxime (CTX)	27 (69.23)	33 (82.5)	7 (25.92)	67 (63.2)	0.01*
Imipenem (IMP)	5 (12.82)	3 (7.5)	0	8 (7.54)	0.095
Meropenem (MEM)	2 (5.12)	1 (2.5)	0	3 (2.83)	0.772
Ceftazidime (CAZ)	8 (20.51)	4 (10)	0	12 (11.32)	0.006*
Aztreonam (ATM)	5 (12.82)	6 (15)	0	11 (10.37)	0.046*
Nalidixic acid (NAL)	8 (20.51)	0	0	14 (13.20)	0.01*
Amoxicillin (AMX)	1 (2.56)	2 (5)	0	3 (0.94)	0.772
Gentamicin (GEN)	15 (38.46)	14 (35)	2 (7.4)	31 (29.24)	0.001*
Ciprofloxacin (CIP)	20 (51.28)	17 (42.5)	20 (74.07)	57 (53.77)	0.740
Ampicillin (AMP)	2 (5.12)	2 (5)	0	4 (3.77)	0.544
Amikacin (AMK)	9 (23.07)	14 (35)	0	23 (21.69)	0.01*
Polymixin B (PMB)	1 (2.56)	0	0	1 (0.94)	1.000
Cefotaxamine+clauvinic acid (CCA)	0	1 (2.5)	0	1 (0.94)	1.000
Ceftriazone (CRO)	0	1 (2.5)	1 (3.7)	2 (1.88)	1.000
Piperacillin+tazobactam (TZP)	10 (25.64)	9 (22.5)	2 (7.4)	21 (19.81)	0.037*

Table 1: Frequency of resistance to antimicrobial agents in DEC isolates from three study groups. *Statistically significant.

**Antibiotic frequencies are presented as absolute numbers (n) with percentage in parentheses.

Groups	Average Age (Years)	Phenotypic		Genotypic
		ESBL Positive by Screening	ESBL Confirmation by DDST	ESBL by PCR
Group 1 (n = 40)	2.17	28 (70)	27 (67.5)	27 (67.5)
Group 2 (n = 40)	1.74	29 (72.5)	27 (67.5)	27 (67.5)
Group 3 (n = 40)	3.88	22 (55)	22 (55)	23 (57.5)
Total (n = 120)	2.59	79 (65.83)	76 (63.33)	77 (64.16)
P value (Group 1,3)	0.061	0.311	0.251	0.485
P value (Group 2,3)	0.166	0.311	0.251	0.485

Table 2: Comparison of ESBL isolation by phenotypic and genotypic methods percentage in parentheses.

***ESBL infection		Age		P value	Odds ratio 95% CI** (lower-upper)
		(< 30 months)	(30-60) months	Group 3 as reference	
(Group 1 and 3)	ESBL+	30	10	0.061	2.455 (0.950-6.339)
	ESBL-	10	40		
(Group 2 and 3)	ESBL+	28	12	0.166	1.909 (0.761-4.788)
	ESBL-	12	28		

Table 3: Multivariable logistic regression showing association of ESBL infection with different age groups.

** CI = confidence interval

***ESBL = extended spectrum beta lactamases.

The genotyping results of ESBL producing isolates obtained by multiplex PCR are shown in Figure 1. Of the total 77 ESBL producers (27, 27 and 23 from group 1, 2 and 3 respectively) detected from 120 E.coli isolates, TEM alone was present in 49 (40.83%), SHV alone in 39 (32.5%), CTX-M alone in 23 (19.16%) and OXA alone in 20 (16.66%) samples as shown in Table 4 (a). The presence of ESBL genes either alone or in combination with other ESBL genes is shown in Table 4 (b) & (c). The presence of TEM and SHV genes alone was also detected in ESBL negative (by combined disk test) isolates.

(a)

Genes	Group1(n=40)	Group2(n=40)	Group3(n=40)	Total (n=120)		P value
TEM	19(47.5)	14(35)	16(40)	49(40.83)	88(73.33)	<0.001*
SHV	14(35)	14(35)	11(27.5)	39(32.5)		
CTX-M	7(17.5)	8(20)	8(20)	23(19.16)	43(35.83)	
OXA	6(15)	7(17.5)	7(17.5)	20(16.66)		

	Phenotypic methods (ESBL)	TEM	SHV	CTX	OXA	TEM + SHtV	TEM + CTX	TEM + OXA	SHV + CTX	SHV + OXA	CTX + OXA	TEM + SHV + CTX + OXA
Group-1 (n = 40)	Positive (n = 27)	3 (11.11)	1(3.70)	1(3.70)	1(3.70)	7 (25.92)	4 (14.81)	4 (14.81)	3 (11.11)	2 (7.4)	0	0
	negative (n = 13)	2 (15.38)	2 (15.38)	0	0	0	0	0	0	0	0	0
P value (Group 3 as reference) #		0.499	0.626	0.775	0.762	1.000	1.000	1.000	1.000	0.675	0.116	1.000
Odds ratio 95% CI ** (lower-upper)		1.357 (0.559-3.292)	1.269 (0.487-3.311)	0.848 (0.275-2.613)	0.832 (0.253-2.737)	1.000 (0.316-3.169)	1.370 (0.286-6.559)	0.778 (0.193-3.137)	0.730 (0.152-3.492)	0.474 (0.082-2.746)	2.111 (1.666-2.676)	2.026 (1.620-2.533)

(b)- Group 1

	Phenotypic methods (ESBL)	TEM	SHV	CTX	OXA	TEM + SHV	TEM+ CTX	TEM + OXA	SHV + CTX	SHV + OXA	CTX + OXA	TEM + SHV + CTX + OXA
Group-2 (n = 40)	Positive (n = 27)	3 (11.11)	0	1 (3.70)	0	8 (29.62)	4 (14.81)	2 (7.4)	4 (14.81)	3 (11.11)	3 (11.33)	0
	negative (n = 13)	0	2 (15.38)	0	0	0	0	0	0	0	0	0
P value (Group 3 as reference) #		0.644	0.469	0.431	1.000	0.606	1.000	1.000	0.432	1.000	1.000	1.000
Odds ratio 95% CI ** (lower-upper)		0.808 (0.326-2.000)	1.420 (0.549-3.673)	1.517 (0.536-4.293)	1.000 (0.316-3.169)	1.347 (0.434-4.180)	1.370 (0.286-6.559)	0.368 (0.067-2.023)	1.000 (0.232-4.310)	0.730 (0.152-3.492)	0.730 (0.152-3.492)	2.026 (1.620-2.533)

(b)- Group 1

# Group 3 (n = 40)	Phenotypic methods (ESBL)	TEM	SHV	CTX	OXA	TEM + SHV	TEM+ CTX	TEM + OXA	SHV + CTX	SHV + OXA	CTX + OXA	TEM + SHV + CTX + OXA
	Positive {n = 22}	4 (18.18)	1 (4.54)	2 (9.09)	0	5 (22.72)	2 (9.09)	2 (9.09)	1 (4.54)	0	0	1 (4.54)
	Negative {n = 18}	1 (5.55)	0	0	0	0	0	0	0	0	0	0

(c)-Group 2

Table 4: Presence/absence of TEM, SHV, CTX-M and OXA genes in E.coli isolates by Multiplex.

PCR *significant P values

** CI=confidence interval

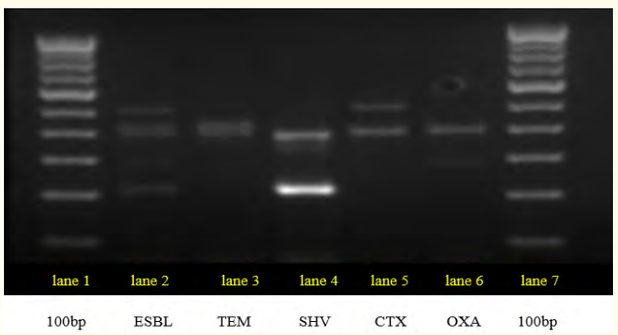

Figure 1: Multiplex PCR products of SHV (214 b p), TEM (431 bp), CTX-M (501 bp) and OXA (296 bp) genes. Lanes 2-6: showing E.coli genes and 16s RNA (401 bp); lane 1 & 7:100 bp size marker.

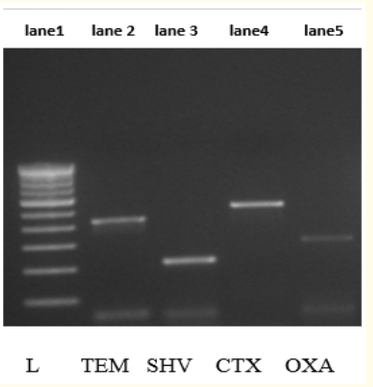

Figure 2: A Uniplex PCR: of SHV (214 b p), TEM (431 bp), CTX-M (501 bp) and OXA (296 bp) genes.

Lane 1: 100bp ladder and Lanes 2-5: showing ESBL genes

Eight isolates of *E.coli* were (6.5%) positive for ESBL production by PCR but negative by combined disc test. Specimens that revealed ESBL production by multiplex PCR were further confirmed by uniplex PCR (Figure 2).

DNA sequence analysis of the PCR product of each of the tested primer pairs by BLAST search showed 96 to 100% identification and query coverage to the Gene Bank database sequences. Nucleotide sequence were deposited in NCBI's BankIt submission database and the following accession numbers were obtained KP973433, KP973432, KP973428, KP973427 etc (Figure 3). No mutations were detected in restriction site of these genes, when we compared our sequences with already existing sequence in NCBI database.

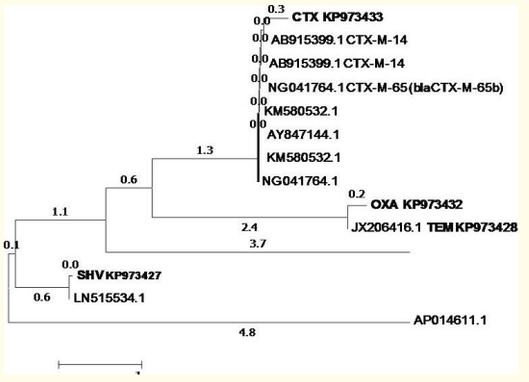

Figure 3: Phylogenetic tree showing evolutionary history using the Neighbor-Joining method.

Discussion

Data regarding the prevalence and composition of various types of extended-spectrum beta lactamases (ESBL) in children under five is sparsely available. Current prevalence of ESBL producing organisms is widely variable globally and reported to be between < 1 percent to 74 percent [17]. This variation may be due to the presence of mobile genetic elements. CTX-M type ESBL is associated with a highly complex genetic structure and harbor ESBL genes and mobile genetic elements which are also found in a variety of plasmids and often carry antibiotic resistance genes.

Majority of isolates in our study were multidrug resistant. The definition most frequently used for multidrug resistant bacteria is 'resistant to three or more antimicrobial classes' [18-23]. Most DEC isolates were predominantly sensitive to polymixin B, ceftriaxone,

amoxicillin & cefotaxime + clavulanic acid. Maximum resistance was seen with cefotaxime (69.23%, 82.5%, 25.9%) followed by ciprofloxacin (51.28%, 42.5%, and 74.07%) and norfloxacin (23.07%, 17.5%, and 37.03%) ($P < 0.05$) in isolates from three groups respectively. It is in agreement with other reported studies [24]. High rates of ESBL positive isolates are recorded in different parts of India, and not restricted to any geographical region [25]. Our study also showed high ESBL production (67.5 percent) each in group 1 and 2 which was slightly higher than our healthy group however; the difference among the groups was not significant. Among neighboring countries, ESBL production in Islamabad and Rawalpindi was found to be 48 and 35 % respectively among *E. coli* isolates ; 9.2 % in Korea and 10.3 % in Arabia [26-28]. It was observed that, children admitted to the hospital receiving antibiotics harbored high ESBL producing *E.coli* similar to children with acute diarrhea, suggesting the survival of resistant strains in the community of this vulnerable population. Further, it probably hints towards the extensive use of antibiotics by clinicians in pediatric age group.

Molecular epidemiology of carriage of ESBL producing DEC as observed in 40 healthy children (57.5%) was higher than cited in other limited studies [29, 30]. The frequency of TEM gene was 47.5%, 40% and 35% in groups 1, 3 and 2 respectively. The existence of SHV gene was higher in group 1 and 2 (35%) in comparison to healthy group (27.55%). The production of CTX and OXA genes was more frequent in group 2 and 3 with 20% and 17.5% each as compared to 17.5% and 15% in group 1. This pattern of occurrence of ESBL genes in our study (TEM followed by SHV, CTX-M and OXA) is similar to other studies [31, 32]. Presence of possibility of other ESBL genes cannot be ignored, as we detected only four common ESBL genes among other 200-300 known genes. Detection of ESBL production by phenotypic and genotypic methods showed marginal difference. The genotypic method has a higher reproducibility as compared to that of phenotypic methods and proves to be a better tool for detecting ESBL producing isolates of diarrheagenic *E.coli.*

Studies from different countries report erratic prevalence of ESBL producing *E.coli* colonization [33-38]. Enhanced pathogenicity of the bacteria carrying beta-lactamase genes increases the mortality risk of infected individuals and poses a threat to the community [39, 40]. TEM and SHV genes have a high rate of occurrence in E.coli as seen in our study [41]. The chance of occurrence of both the genes is almost similar, since SHV-1 shares 68 percent similarity of its amino acids with TEM-1 and has a similar overall structure [42, 43]. Therefore, TEM and SHV genes (P value highly significant) prove to be useful tools for the molecular screening of ESBL positive samples in hospital settings. Incorrect identification of antibiotic resistance; frequently leads to uncontrolled selection and spread of such resistant genes amongst the gut microflora; hence a continuous vigilance and surveillance of prevention of antibiotic resistance in hospitals, becomes a priority.

In order to increase the accuracy of the results, sequencing was performed with both forward and reverse primers of ESBL genes. Absence of mutation in this population is suggestive of probable transfer of same copy of ESBL genes amongst the various isolates by mobile genetic elements.

Conclusion

ESBL producing DEC is a major concern in children suffering from diarrhea. The pediatric population is at a higher risk of acquiring multidrug resistance flora, due to limitation in therapeutic options and restricted prescription policy of harsher antibiotics especially the use of cephalosporins. The results of this study demonstrated a need for heightened awareness regarding the increasing frequency of these highly resistant isolates as reservoirs in pediatric population and their potential impact on transmission to community and hospital environment. This observation also underscores the need to improve microbiological diagnostic facilities and antibiotic resistance surveillance in resource poor settings; to be able to promptly revise antibiotic regimens and avoid emergence of resistance.

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