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Original Article

Detection of virulence genes in ESBL producing, quinolone resistant commensal *Escherichia coli* from rural Indian children

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Abstract

Introduction: Extended-spectrum β-lactamase producing commensal *Escherichia coli* are considered as a reservoir of antibiotic resistance genes that may be transmitted in the community. This study aimed to determine the genes coding for ESBLs, plasmid mediated quinolone resistance and virulence markers in commensal *E. coli* isolated from healthy school children.
Methodology: ESBL producing *E. coli* isolates (n = 47) were obtained from 529 fecal samples of healthy school children from a rural area in central India. Multiplex PCR was used to detect the genes coding for cephalosporin and quinolone resistance, for virulence *fluA*, *fluB*, stx_1, stx_2, *eae, bfp, lt, stII, virF, ipaH, daaE, aafII* and phylogenetic groups.
Results: Of the 47 ESBL producing *E. coli,* 41 were positive for CTXM-15, 23 for TEM-1, 8 for OXA-1and a single for SHV-12. For plasmid-mediated quinolone resistance, all the 47 isolates carried the *aac*(6')-*ib-cr* gene, and amongst them18 were *qnrS* positive. Virulence gene, *fluA* was detected in 32,whereas *eae* in 14, *daaE* in 7 and *fluB* in 1. In 10 isolates, *fluA* and *eae* and in 7, *fluA* and *daaE* co-existed. Of the 47 *E. coli* isolates, 18 were grouped into the phylogenetic group B2, 17 in D and 12 in A. The proportion of isolates positive for *fluA* gene in the phylogenetic group B2 (18/18), was significantly higher than in group A (7/12) and D (6/17).
Conclusion: Commensal *E. coli* in healthy children in rural India may serve as reservoirs of resistance towards cephalosporins and fluoroquinolones and virulence coding genes for urinary tract and diarrheal infections.

Key words: Antibiotic resistance; ESBLs; virulence factors; commensal *Escherichia coli;* children; quinolones.

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Introduction

Escherichia coli form part of the bacterial flora of the human gastrointestinal tract, and also act as reservoirs of antibiotic resistance coding genes [1]. *E. coli* cause infections such as urinary tract infections (UTIs), diarrheal diseases, neonatal meningitis, community acquired infections and health- care associated infections. Several virulence mechanisms are associated with the pathogenesis of *E. coli*. Anautotransporter (AT) protein Ag43a coded by the *fluA* and *fluB* gene have been detected in *E. coli* [2]. The *fluA* has a high level of auto aggregative properties responsible for the persistence of infection in the urinary bladder, while *fluB* is responsible for the initial colonization of *E. coli* in the urinary tract [3]. These auto transporter proteins enable adherence to and invasion of uroepithelial cells and enhance biofilm formation [4].

Diarrheagenic *E. coli* are classified in six categories. Among these, the diffuse adherent *E. coli* (DAEC) has the capacity to cover the surface of the enterocyte due to Dr adhesin family protein coded by

daaE gene. Another virulence factor, the intimin protein coded by *eae* gene enables the *E. coli* to adhere intimately and cause attachment and effacing (A/E) of the enterocytes [5]. The other categories are the enteropathogenic, enterotoxigenic, enterohemorrhagic, and enteroinvasive *E. coli*.

Cephalosporins and quinolones are commonly used empirically as a treatment of choice for gram negative bacterial infections including those caused by *E. coli*. Plasmid-encoded Extended-Spectrum beta-lactamases (ESBLs) impair the effectiveness of third generation cephalosporins. Among ESBLs, the CTX-M-15 gene was reported globally in commensal and clinical isolates of *E. coli* [6]. The *qnr* genes, *aac*(6')-*lb-cr, oqxAB* and *qepA* genes code for plasmid mediated quinolone resistance (PMQR). Enterobacteriaceae isolates from low and middle income countries are reported to harbour PMQR coding genes co-existing with genes coding for ESBLs [7]. The aim of this study was to detect genes coding for ESBLs, PMQRs and virulence factors associated with UTIs and diarrhea in commensal *E. coli* isolated from healthy school children from rural India and further to classify such *E. coli* into phylogenetic groups.

Methodology

A total of 47 non duplicate ESBL producing *E. coli* isolates from fecal samples of 529 healthy children living in the demographic surveillance site (DSS) attached to Ruxmaniben Deepchand Gardi Medical College, in Central India were used for this study. The details of data collection, identification, antibiotic susceptibility testing (AST) for these 47 ESBL producing *E. coli* isolates have been reported earlier [8]. Minimum inhibitory concentration (MIC) was determined by agar dilution method for cefotaxime, ceftazidime and ciprofloxacin according to CLSI guidelines [9]. *E. coli* ATCC 25922 was used as a control. The ESBL producing commensal *E. coli* isolates were selected for further study.

The DNA from ESBL producing *E. coli* was extracted by alkaline lysis method [10]. PCR amplification and identification of ESBL encoding genes (CTX-M, OXA, TEM, SHV) and plasmid mediated quinolone resistance encoding genes (*qnr -A,B,S,oqxAB,qepA,aac(6')ib-cr)* was done using previously described primers [11,12]. Virulence factors for UTI were detected by using primers for *fluA, fluB* genes and a multiplex of 10 primers was used for the detection of diarrheagenic *E. coli* pathotype (stx_1,stx_2,*eae,bfp,lt,stII,virF,ipaH,daaE,aafII*) [3,13]. After PCR amplification, agarose gel electrophoresis with 2% agarose was performed and the amplified gene products were visualized by gel documentation system. The ESBL producing isolates were phylo grouped by multiplex PCR based on *chuA, yjaA* and *TspE4C2* genes [14]. The nucleic acid sequences were submitted to Gen-Bank (GenBank accession numbers KR233797-KR233805).

Data management and statistical analysis

Data was entered in SPSS Statistics 20.0 (SPSS Inc., Chicago, IL, USA) and were analysed using descriptive statistics, frequencies and bivariate analysis (cross-tabulations). Chi-square test with Yates' correction was used to compare the results of phylogenetic grouping and presence of *flu A* gene. A significance level of $p \leq 0.05$ was used. The study was approved by the ethics committee of R.D.Gardi Medical College, Ujjain (No. 41/2007).

Results

All the 47 ESBL producing isolates of commensal *E. coli* had a MIC of > 512 µg/ml to cefotaxime and the MIC range for ceftazidime was found to be between 64-512µg/ml. Resistance to ciprofloxacin was observed in 23 (49%) out of 47 isolates. Analysis of the genes coding for cephalosporin and quinolone resistance among the 47 ESBL producing *E. coli* is presented in Table 1. The CTXM-15 was the most commonly

Table 1. Cephalosporin and quinolone resistance coding genes detected from ESBL producing commensal *E. coli* isolated from children (3-14 years) in Ujjain, Central India.

Cephalosporin resistance coding genes	n (%)	Quinolone resistance coding genes	n (%)	Cephalosporin and quinolone coding genes	n (%)
CTXM-15	41 (87)	*qnr* S	18 (28)	CTXM-15+*qnr*S	13 (28)
TEM-1	23 (49)	*aac (6')-ib-cr*	47 (100)	CTXM-15 + *qnr*S + *aac(6')- ib-cr*	13 (28)
SHV-12	1 (2)			CTXM-15 + TEM-1 + OXA-1	13 (28)
OXA-1	8 (17)				
CTXM-15 +TEM-1	22 (47)				
CTXM-15 + OXA-1	7 (15)				

detected ESBL coding gene (41/47). All commensal *E. coli* isolates (n = 47) in our study carried *aac*(6')-*ib-cr* gene. However, only half of them (49%) showed resistance to ciprofloxacin. As seen in Table 2, the *fluA* gene was detected in 32/47 (68%) *E. coli* isolates. A total of 13 of the 47 ESBL producing *E. coli* showed the presence of *eae* gene (28%) and in 10 (21%) isolates, *eae* gene co-existed with *fluA* gene. The distribution of virulence marker genes in different phylogenetic groups is shown in Table 2. None of the *E. coli* isolates belonged to phylogenetic group B1. Most of the isolates were grouped in either B2 (38%) or D (36%). The *fluB* gene was found in a single isolate belonging to group A.

Discussion

The present study investigated genes coding for ESBLs, PMQRs and virulence factors *fluA* and *fluB* associated with UTIs and diarrhea in ESBL producing commensal *E. coli* isolated from healthy school children. All the 47 ESBL producing *E. coli* isolates had high MIC values (≥ 512µg/ml) for cefotaxime, ceftazidime and some of the isolates showed co-resistance to ciprofloxacin. These findings are in line with our earlier studies exploring antibiotic resistance in the same study area, wherein we reported high MIC (> 512µl/ml) to cefotaxime and ciprofloxacin (256µl/ml) [15].

The common mechanism of resistance to third generation cephalosporins in *E. coli* is ESBL production coded by CTX-M. Among the CTX-M gene, CTX-M-15 subtype detected from commensal *E. coli* was reported earlier from the study area [15]. The TEM group of beta-lactamase coding genes, subgroup TEM-1 was not found to be associated with ESBL production. In our study, 49% (n= 23/47) isolates harboured the TEM-1 gene and in almost all (22/23, 96%) of the isolates harbouring this gene, it co-existed with CTX-M-15 gene. Only one isolate harbored TEM-1, CTX-M-15 and OXA-1 genes together.

The *aac*(6')-*ib-cr* gene codes for aminoglycoside acetyltransferase that confers reduced susceptibility to

Table 2. Distribution of virulence genes with in phylogenetic groups among ESBL producing commensal *E. coli* isolates from healthy children in Ujjain; Central India.

	A (n=12)							B2 (n=18)							D (n=17)						
Cephalosporin and quinolone resistance genes	***Flu A***	***eae***	***daaE***	***Flu A + eae***	***Flu A + daaE***	***Flu A + Flu B + eae***	***Not detected***	***Flu A***	***eae***	***daaE***	***Flu A + eae***	***Flu A + daaE***	***FluA + eae + daaE***	***Not detected***	***Flu A***	***eae***	***daaE***	***Flu A + eae***	***Flu A + daaE***	**Not detecte**	**Total**
CTX-M-15 + *aac(6')- ib-cr*	1	1	-	1	-	1	1	1	-	-	-	-	-	-	-	-	-	2	-	3	**11**
CTX-M-15 + OXA + *aac(6')- ib-cr*	-	-	-	-	-	-	1	2	-	-	-	-	-	-	-	-	-	-	-	-	**3**
CTX-M-15 + TEM-1 + *aac(6')- ib-cr*	3	1	-	-	-	-	2	2	-	-	2	2	-	-	2	-	-	-	-	1	**15**
CTX-M-15 + qnrS + *aac(6')- ib-cr*	-	-	-	-	-	-	-	-	-	-	-	1	1	-	-	1	-	1	-	-	**4**
CTX-M-15 + TEM-1 + qnrS + *aac(6')- ib-cr*	-	-	-	-	-	-	-	3	-	-	-	1	-	-	-	2	-	-	-	-	**6**
CTX-M-15 + SHV-12 + qnrS + *aac(6')- ib-cr*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**-**
CTX-M-15 + TEM-1 + OXA-1 + *aac(6')- ib-cr*	-	-	-	-	-	-	-	1	-	-	-	1	-	-	-	-	-	-	-	-	**2**
CTX-M-15 + OXA-1 + qnrS + *aac(6')- ib-cr*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	**1**
CTX-M-15 + TEM-1 + OXA-1 + qnrS + *aac(6')- ib-cr*	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	**1**
CTX-M-15 + SHV-12 + OXA-1 + *aac(6')- ib-cr*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	**1**
Total	**4**	**2**	**-**	**1**	**-**	**1**	**4**	**10**	**-**	**-**	**2**	**5**	**1**	**-**	**3**	**3**	**-**	**3**	**-**	**5**	**44 ***

There were no isolates which carried only cephalosporins and quinolones resistance ; * *flu A* was present in one isolate but it didn't carry any other virulence marker tested, as well as it and virulence marker tested was not detected in two strains that carried combinations of quinolone resistance genes, *qnrS* + *aac(6')-ib-cr;* There were three isolates that carried combination of quinolone resistance genes qnrS + *aac(6')- ib-cr* one of which carried only *flu A* and the other two didn't carry any of the tested virulence marker; Hyphen (-) indicates absence of relevant entry.

ciprofloxacin. Our findings are in accordance with Park *et al*. who reported the presence of *aac*(6')-*ib-cr* gene in almost equal proportions of ciprofloxacin susceptible and resistant isolates from the United States [16]. The *qnrS* gene was detected in 18 (38%) out of the 47 isolates and a single isolate had *qnrB* gene. Among the *qnrS* gene carrying isolates, all 18 of them had *aac*(6')-*ib-cr;* whereas13 of them carried CTX-M-15 gene. Fihman *et al.* reported detection of *aac*(6')-*Ib-cr* associated with CTX-M-15 enzyme in *E. coli* isolates from inpatients from a French hospital [17]. Fortini *et al.* reported that a plasmid named as pPGRT46 isolated from commensal *E. coli* from Nigeria supported the spread of the CTX-M-15 and *qnrS1* genes [18]. The CTX-M-15 gene environment in this plasmid consisted of the IS*Ecp1* inserted into a Tn*3* transposase gene and *tnpA, strA, strB, sul2* genes. Additionally *In4*-type class 1 integron with the *dfrA14* gene cassette, flanked by the *uvp1* resolvase gene was also present. Further, *qnrS1* gene environment consisted of IS*2* along with *tniR* gene [18]. Carriage of CTXM-15 along with PMQR coding genes carried by plasmids capable of exchange between strains, may lead to dissemination of these resistance coding genes in the community.

The distribution of ESBL producing commensal *E. coli* carrying *fluA* gene in phylogenetic group B2 (n = 18) was significantly higher when compared to D (n = 6, p =0.005) and A (n = 6, p = 0.0001). Qin *et al.* in 2013 reported a significantly higher prevalence of *fluA* gene in UPEC isolates than in commensal strains [19]. Lüthje and Brauner reported a presumed link between *fluA* gene and fluoroquinolone resistance in uropathogenic *E. coli* (UPECs) isolated from adult patients with urosepticemia from Sweden [20]. In our study, a total of 49% (n = 23/47) of commensal *E. coli* isolates showed resistance to quinolones and among these 65% (n = 15/23) isolates carried *fluA* gene. Lüthje and Brauner observed a significant association of *flu* genes with *E. coli* isolated from recurrent UTI infections and linkage between Ag43 proteins coded by *flu* gene with intracellular persistence of *E. coli* causing UTI infections [21]. Ramos *et al.* reported a high prevalence of agn43 coded by *flu* genes, with *fluB* gene being less commen compared to *fluA* genes, among the *E. coli* isolates causing UTI in pregnant women from several countries [22]. In our study *fluB* gene, associated with reduced cell aggregation and less biofilm production, was carried by a single commensal *E. coli* isolate. Vollmerhausen and Katouli reported agn43b, coded by *flu B* gene was prevalent among *E. coli* isolates in hospitalized children with urinary tract infections from Australia [23]. Our study tested only two extra-intestinal pathogenic *E. coli* (ExPEC) associated virulence markers and it is a limitation of our study. We are aware that there are other virulence factors also associated with ExPEC. However, due to financial constraints, we could not include other virulence markers associated with ExPEC.*E. coli* has been recognised as both a harmless commensal and a versatile pathogen responsible for many of human infections. UTIs are common in children and *E. coli* has been reported as the most common uropathogen causing community acquired UTI in Indian children [24,25]. Most of the pathogenic bacteria, which cause UTI, are from the host's own commensal flora and enter the bladder via the urethra. Uroepithelial adherence is critical for establishment of UTI. Increased incidence of community acquired UTIs in children, caused by ESBL producing organisms including *E. coli* from 15% in 2010 to 65% in 2013has been reported from one hospital in South India. However, in that study the presence of any virulence markers was not reported [25]. In our study, *fluA* gene that codes for Ag43a protein responsible for the persistence of infection in the urinary bladder was detected in ESBL producing commensal *E. coli* isolated from rural children. Further, in our study, two genes associated with diarrhea, *daaE* gene that codes for dr adhesion and *eae* gene, which codes for intimin protein that is necessary for intimate attachment to host epithelial cells co-existed with *fluA* gene among commensal *E. coli* isolates. Diarrhea is still an important cause of death among children in low and middle income countries including India [26]. Madhya Pradesh, a central Indian state, where this study has been conducted, recently reported an increasing number of acute diarrheal cases and deaths due to diarrhea [27]. A study conducted in pharmacies and hospitals of this geographical region reported that 71% of children with diarrhea (aged 1 month-12 years) were prescribed antibiotics [28]. This is not according to guidelines and have implications for resistance development and spread. Horizontal transfer of genetic elements carrying genes that codes for virulence factors as well as antibiotic resistance can occur within the same or across different bacterial species and genera. This may lead to the spread of community acquired urinary tract and diarrhea infections potentially caused by resistant bacteria, in the study area.

Conclusions

The presence of urovirulence factors in the commensal *E. coli* together with the co-existence of resistance to cephalosporins and quinolones is evident from our study. Commensal *E. coli* in healthy children

in rural India may thus serve as reservoirs of resistance to cephalosporins and fluoroquinolones and virulence markers coding for urinary tract and diarrheal infections. These findings stress the need to review antibiotic prescribing policies in health-care settings and indicate the need of putting in place antibiotic stewardship programmes to control the spread of antibiotic resistant bacteria in the community.

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Authors Contributions

RM, CSL, SC and VD initiated the study. RM, SC, CSL, HS, AP and SS participated in developing the concepts, the design and the planning of the study. RM, HS, SC and SS did microbiology and molecular biology laboratory work. SS, SC, AP, RM, VD, HS, AJT and CSL were involved in analysis and interpretation of data. SS, SC and RM prepared the first draft. AP, AJT and CSL revised the paper critically for substantial intellectual content. All authors have read and approved the final manuscript.

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