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Antimicrobial susceptibility, risk factors and prevalence of *bla* cefotaximase, temoneira, and sulfhydryl variable genes among *Escherichia coli* in community-acquired pediatric urinary tract infection

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Abstract:

INTRODUCTION: The emergence of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* has become an important challenge among pediatric patients with community-acquired urinary tract infection (UTI).

OBJECTIVES: The aim of this study was to assess the antimicrobial susceptibility patterns, associated risk factors and to survey the frequency of *bla* cefotaximase (CTX-M), *bla* temoneira (TEM), and *bla* sulfhydryl variable (SHV) genotypes in ESBL-producing *E. coli* isolated from children with community-acquired UTI.

METHODS: This was a prospective study conducted from November 2012 to March 2016 in a tertiary care center. *E. coli* isolated in urine cultures from children aged ≤18 years was identified and confirmed for ESBL production. ESBL-positive strains were screened for ESBL encoding genes. Chi-square test and Fisher's exact test were used to compare the difference in antibiotic susceptibility with respect to ESBL positive and negative, and binary logistic regression was used to identify the risk factors associated with ESBL production.

RESULTS: Among 523 *E. coli* isolates, 196 (37.5%) were ESBL positive, >90% were resistant to cephalosporins, and 56% were resistant to fluoroquinolones. Least resistance was observed for imipenem, netilmicin, and nitrofurantoin (2%, 8.6%, 15.3%). Association between ESBL production and drug resistance was significant for ceftazidime ($P < 0.001$), cefixime ($P < 0.001$), cefotaxime ($P = 0.010$), ceftazidime-clavulanic acid ($P < 0.001$), levofloxacin ($P = 0.037$), and gentamicin ($P = 0.047$) compared to non-ESBL *E. coli.* CTX-M gene was the most prevalent (87.5%), followed by TEM (68.4%) and SHV (3.1%). Previous history of UTI and intake of antibiotics were the common risk factors.

CONCLUSION: ESBL-producing *E. coli* from community-acquired pediatric UTI carries more than one type of beta-lactamase coding genes correlating their increased antibiotic resistance. Aggressive infection control policy, routine screening for detecting ESBL isolates in clinical samples, and antimicrobial stewardship are the keys to prevent their dissemination in community settings.

Key words:

Cefotaximase, community-acquired pediatric urinary tract infection, extended-spectrum beta-lactamase, sulfhydryl variable, temoneira

Introduction

Escherichia coli is a common pathogen causing community-acquired urinary tract infections (UTIs) in adults as well as in pediatric age groups. In recent studies, *E. coli* is the most frequently isolated extended-spectrum beta-lactamases (ESBL) carrying bacterium.[1] ESBL are enzymes capable of hydrolyzing the oxyimino-cephalosporins and monobactams. They are encoded by plasmids generally derived from cefotaximase (CTX-M), temoneira (TEM), or sulfhydryl variable (SHV).[2] The emergence of ESBL as an important cause of transferable

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drug resistance in *E. coli* is a serious problem in both hospital and community settings.[3]

Extensive use of extended-spectrum antibiotics is one of the most important factors associated with high prevalence of ESBL. Since the antibiotic use varies in different geographical regions, it can cause variation in the prevalence of ESBL genotypes. During the 1990s, SHV and TEM types were dominant among ESBL all over the world and CTX-M-producing organisms were rarely isolated. ESBL production in *E. coli* has significantly increased in numerous countries including India.[3,4] Studies have shown that awareness of risk factors in ESBL-producing *E. coli* UTI infections can minimize the complications associated with UTI, reduce the burden of medical expenses and development of antibiotic resistance, and enhance the clinical outcome among the pediatric patients.

To the best of our knowledge, no long-term prospective epidemiological surveillance studies have been carried on ESBL-producing *E. coli* strains among pediatric population in the community settings in India. Very few studies have described the risk factors and antibiotic resistance patterns of community-acquired pediatric UTI caused by ESBL-producing bacteria in India.[3,4] Thus, the aim of this study was to evaluate the resistance of the ESBL producing *E. coli* strains to commonly used antibiotics, to assess the risk factors associated with ESBL production, and to detect the presence of the three common ESBL genes: TEM, CTX-M, and SHV using polymerase chain reaction (PCR) method. The results of the present study might be valuable to both health professionals and the scientific community and may aid the current understanding of the trends in UTI caused by *E. coli.*

Methods

Setting

The present study was carried out in the clinical microbiology laboratory of a tertiary care center in Kerala, India. The study was conducted after obtaining approval from the Institutional Ethics Committee and informed consent from either of their parents.

Study design

It is a prospective microbiological study of *E. coli* isolates from urine specimens of pediatric patients with suspected community-acquired UTI who attended the outpatient department between November 2012 and March 2016.

Inclusion criteria

Children in the age group 3 months to 18 years with culture-proven UTI and satisfying the definition of community-acquired UTI were included in the study. Community-acquired UTI was defined as infection detected at hospital admission or within the first 48 h of admission.

Exclusion criteria

Children who satisfy definitions of health care-associated infection as outlined below:[5,6]

(1) Children who develop UTI during hospitalization (>48 h) or within 48 h of hospital discharge. (2) Children hospitalized in any hospital or nursing home during the 90 days preceding admission. (3) Children having a chronic medical condition requiring frequent medical visits as outpatient or inpatient. (4) Children receiving intravenous therapy or specialized wound care at home. (5) Patient receiving hemodialysis treatment or antineoplastic chemotherapy in 30 days before the infection. (6) Patient residing in a nursing home or transferred from another hospital.

Sample collection and processing

Urine samples were obtained by either of the following methods, i.e., midstream catch or bladder catheterization and cultured. *E. coli* on isolates was identified by conventional microbiological methods and according to the criteria of the Clinical and Laboratory Standards Institute (CLSI 2010) guidelines.[7,8]

Antibiotic sensitivity test was performed for *E. coli* by standard Kirby-Bauer disc diffusion method.[9] The following standard antibiotic discs (mcg) were used: ampicillin (10), cefixime (30), gentamicin (10), netilmicin (30), co-trimoxazole (25), ciprofloxacin (5), norfloxacin (10), levofloxacin (5), doxycycline (30), nitrofurantoin (300), and imipenem (10). Screening test for ESBL production was performed using cefotaxime (Ctx) and ceftazidime (CAZ) tested in combination with clavulanic acid. ESBL production was confirmed by phenotypic confirmatory double disc test and triple ESBL detection Ezy MIC™ Strip.[10]

Imipenem resistance was confirmed by E-strip method (imipenem with and without ethylene diamine tetraacetic acid [EDTA] Ezy MIC™ Strips EM078). All culture media and antibiotic discs were procured from HiMedia Laboratories, Mumbai, India. Results were interpreted according to the CLSI 2010 guidelines.[7] Quality control was performed using CLSI recommended *E. coli* ATCC 25922 and *E. coli* 35218 strains. The confirmed ESBLs positive *E. coli* isolates were kept in Luria-Bertani broth with glycerol and preserved at – 80°C for molecular studies.

DNA extraction and identification of *bla* cefotaximase, *bla* temoneira, and *bla* sulfhydryl variable genes by polymerase chain reaction

Confirmed ESBL-producing isolates were characterized for the presence of *bla* CTX-M, *bla* TEM, and *bla* SHV genes by PCR. Preparation of template DNA: *E. coli* isolate preserved in Luria-Bertani broth with glycerol was subcultured onto MacConkey agar. After overnight incubation, a single colony of each organism was inoculated into 5 ml of Luria-Bertani broth (HiMedia diagnostic, Mumbai) and incubated for 24 h at 37°C. 50 µl of this overnight culture was added to 450 µl of 1X TE (Tris-HCl and EDTA) buffer. This was incubated at 95–97°C in dry bath for 15 min and then kept in ice for 10 min. The DNA thus obtained were kept at –20°C.

Bacterial genes associated with ESBL production were detected by PCR amplification of target genes by using specific primers in programmable thermal cycler (MJ Research PTC-200, Bengaluru).[11,12] Table 1 shows the primer sequences and specific thermal cycling conditions of CTX, TEM, and SHV genes. PCR mixtures were prepared using 2 µL template DNA, 28 µL PCR master mix (sterile distilled water 22.2 µL, Taq DNA polymerase 0.2 µL, Taq buffer with MgCl2 3 µL [GeNei,

Table 1: Primer sequences and specific thermal cycling conditions of cefotaximase, temoneira, and sulfhydryl variable genes

Target gene	Primer sequence	Thermal cycling conditions	PCR product size (bp)	Reference
bla CTX-M	ACGTTAAACACCGCCATTCC TCGGTGACGATTTTAGCCGC	95°C 5 min (1 cycle) 94°C 15 s, 60°C 1 min 72°C 1.3 min (30 cycles) 72°C 4 min (1 cycle)	356	[11]
bla TEM	CTCACCCAGAAACGCTGGTG ATCCGCCTCCATCCAGTCTA	95°C 5 min (1 cycle) 94°C 15 s, 63°C 1 min 72°C 1.3 min (30 cycles) 72°C 4 min (1 cycle)	569	[11]
bla SHV	ATTTGTCGCTTCTTTACTCGC TTTATGGCGTTACCTTTGACC	94°C 5 min 94°C 30 s (30 cycles) 52°C 30 s, 72° 50 s 72°C 10 min	1018	[12]

CTX-M = Cefotaximase, TEM = Temoneira, SHV = Sulfhydryl variable, PCR = Polymerase chain reaction

India], dNTP 0.6 µL, 1 µL of each 10 pm primer). Primers were obtained from BioServe Technologies, Hyderabad. 15 µl of the resulting PCR products were mixed with 10 µl of loading dye and analyzed by electrophoresis in 1.5% agarose gels (HiMedia, India) containing 1x Tris-acetate EDTA (1X TAE buffer) and 10 µl of ethidium bromide and visualized with the help of gel documentation system (Bio-Rad, India). The PCR band observed at 569 bp (CTX), 356 bp (TEM), and 1018bp (SHV).

In addition, clinical data and risk factors were analyzed for their predictability for colonization by an ESBL-producing organism.

Statistical analysis

The data were analyzed using Statistical Package for Social Sciences software version 16 (Chicago, SPSS Inc). The collected information was summarized using frequency, percentage, mean, and standard deviation (descriptive statistics). To compare the difference in antibiotic susceptibility with respect to ESBL positive and negative, Chi-square test and Fisher's exact test were used. To find the strength of association between antibiotic sensitivity and ESBL positive and negative groups, odds ratio (OR) and 95% confidence interval (CI) were computed. Binary logistic regression was used to identify the risk factors associated with ESBL production. A $P < 0.05$ was considered statistically significant.

Results

A total of 523 children with culture-proven *E. coli* community-acquired UTI were included in the study. Of this, 319 (60.9%) were boys and 204 (39.1%) girls, with a mean age of 31.19 ± 1.44 months. ESBL positivity was seen in 196 (37.5%), of which 131 (66.8%) were boys and 65 (33.2%) girls. The mean ages of ESBL producers and nonproducers were 30.62 ± 2.21 and 30.92 ± 1.68 months, respectively, with no statistical significance ($P = 0.537$). Statistical difference between male and female gender with regard to ESBL production was significant (Chi-square 4.49; $P = 0.034$).

Table 2 allows the comparison of antibiotic resistance patterns of ESBL positive and negative community-acquired *E. coli.* The association between ESBL production and drug resistance was significant for CAZ (OR 20.15, $P < 0.001$), cefixime (OR 4.21, $P < 0.001$), Ctx (OR 2.46, $P = 0.010$), ceftazidime-clavulanic acid (OR 0.321, $P < 0.001$), levofloxacin (OR 0.674, $P = 0.037$), and gentamicin (OR 1.487, $P = 0.047$) when compared to non-ESBL *E. coli.* ESBL-positive *E. coli* showed more than 90% of resistance to cephalosporin group of antibiotics and 56% against ciprofloxacin and norfloxacin. Resistance against doxycycline, gentamicin, and levofloxacin was less than 36%. Least resistance was observed for imipenem, netilmicin, and nitrofurantoin.

Disc diffusion test revealed that 4 (2%) of the ESBL-positive isolates were resistant to imipenem. This was confirmed using imipenem-EDTA strip method. MIC range was 4–8 µg/ml for three of the isolates. One isolate showed MIC >256 µg/ml.

Multivariate analysis by binary logistic regression showed the following factors being significantly associated with *E. coli* resistance: history of previous UTI (OR 1.10, 95% CI, 0.312–3.880, $P < 0.001$), history of previous intake of antibiotics such as nitrofurantoin (OR 11.49, 95% CI, 1.484–89.084, $P < 0.001$) and quinolones (OR 1.15, 95% CI, 0.316–4.257, $P < 0.001$). The same is shown in Table 3 which includes other risk factors studied.

CTX-M was the most prevalent gene (87.5%), followed by TEM (68.4%) and SHV (3.1%) among the ESBL producers. About 48.5% of ESBL isolates were shown to have both TEM and CTX-M genes, 2.6% possessed CTX and SHV, and 2% had TEM and SHV. Two percent of the isolates were shown to have CTX, TEM, and SHV [Figure 1a-c and Table 4].

Table 5 shows association between antimicrobial resistance and gene expression in ESBL producing *E. coli.* In most of the cases, there were not any significant differences regarding genes expression and antibiotic resistance profile. Higher resistance to ampicillin, cefixime, Ctx, and Caz was found in CTX-positive group. Resistance to cefixime, Ctx, Caz, ciprofloxacin, and co-trimoxazole was observed more in TEM-positive isolates.

Discussion

Worldwide, the incidence of community-onset adult UTI caused by ESBL-producing bacteria is rising, but very few studies have described the epidemiology of community-acquired pediatric

Table 2: Comparison of antibiotic resistance patterns of extended-spectrum beta-lactamase producing and nonextended-spectrum beta-lactamase *Escherichia coli* isolates (*n*=523) in community-acquired pediatric urinary tract infection

Antibiotic	ESBL		χ^2	*P*	OR	95% CI
	Negative (*n*=327)	Positive (*n*=196)				
Amp						
Resistant	214	135	0.651	0.420		
Sensitive	113	61				
Cfm						
Resistant	247	182	24.94	<0.001*	4.21	2.31-7.66
Sensitive	80	14				
CTX						
Resistant	296	188	5.175	0.010*	2.46	1.10-5.46
Sensitive	31	8				
Caz						
Resistant	249	193	46.65	<0.001* (Fisher's exact)	20.15	6.26-64.82
Sensitive	78	3				
Cac						
Resistant	289	139	25.13	<0.001*	0.321	0.203-0.50
Sensitive	38	57				
Cip						
Resistant	189	109	0.239	0.625		
Sensitive	138	87				
Cot						
Resistant	173	113	1.11	0.291		
Sensitive	154	83				
Do						
Resistant	109	70	0.309	0.579		
Sensitive	218	126				
Gen						
Resistant	79	63	3.949	0.047*	1.487	1.004-2.20
Sensitive	248	133				
Ipm						
Resistant	0	4	2.416	0.302		
Sensitive	324	192				
Lev						
Resistant	135	63	4.350	0.037*	0.674	0.46-0.97
Sensitive	192	133				
Net						
Resistant	37	17	0.924	0.337		
Sensitive	290	179				
Nit						
Resistant	50	30	0.000	0.996		
Sensitive	277	166				
Nx						
Resistant	165	110	1.577	0.209*	1.256	0.88-1.79
Sensitive	162	86				

*Significant at 5%. Amp = Ampicillin, Cfm = Cefixime, Ctx = Cefotaxime, CAZ = Ceftazidime, Cac = Ceftazidime-clavulanic acid, Cip = Ciprofloxacin, Cot = Co-trimoxazole, Do = Doxycycline, Gen = Gentamicin, Ipm = Imipenem, Lev = levofloxacin, Net = Netilmicin, Nit = Nitrofurantoin, Nx = Norfloxacin, OR = Odds ratio, CI = Confidence interval, ESBL = Extended-spectrum beta-lactamase

UTI caused by ESBL-producing bacteria in India.[3,4] The present study (*n* = 523) has shown that 37.5% of community-acquired *E. coli* isolates were ESBL producers. Similar results were reported from Turkey (*n* = 344) and Saudi Arabia (*n* = 207), in which the prevalence of ESBL-positive *E. coli* was 41.4% and 44%, respectively, in community-acquired UTI among children.[13,14]

Community-acquired ESBL *E. coli* showed high resistance rate to most of the currently used antimicrobial agents such as ampicillin (68.8%), cephalosporins including third-generation (>90%), quinolones specifically ciprofloxacin and norfloxacin (>50%), and co-trimoxazole (57.6%) but comparatively less resistance rate to gentamicin (32.1%), levofloxacin (32.1%), nitrofurantoin (15.3%), netilmicin (8.6%),

Table 3: Risk factors associated with extended-spectrum beta-lactamase production in *Escherichia coli* isolates (*n*=523) from community-acquired pediatric urinary tract infections by logistic regression

Risk factors	ESBL		χ^2	*P*	Adjusted OR	95% CI
	Negative (*n*=327)	Positive (*n*=196)				
Previous stay	38	92	81.839	<0.001	0.75	0.316-1.780
Circumcision	56	46	3.142	0.076	0.447	0.225-0.890
Recurrent UTI	13	36	29.89	<0.001	0.483	0.174-1.343
Previous UTI	37	87	74.10	<0.001	1.101	0.312-3.880
Previous ampicillin	78	173	203.70	<0.001	0.160	0.085-0.303
Previous cephalosporin	21	142	249.03	<0.001	0.015	0.003-0.068
Previous nitrofurantoin	21	69	71.25	<0.001	11.499	1.484-89.084
Previous quinolones	23	67	63.40	<0.001	1.159	0.316-4.257

UTI = Urinary tract infection, OR = Odds ratio, CI = Confidence interval, ESBL = Extended-spectrum beta-lactamase

Table 4: Frequency of extended-spectrum beta-lactamase genes isolated in *Escherichia coli* isolates (*n*=523) from community-acquired pediatric urinary tract infections

Name of gene	Frequency (%)
CTX	172 (87.5)
TEM	134 (68.4)
SHV	6 (3.1)
CTX and TEM	95 (48.5)
CTX and SHV	5 (2.6)
TEM and SHV	4 (2)
CTX, TEM, and SHV	4 (2)

CTX = Cefotaximase, TEM = Temoneira, SHV = Sulfhydryl variable

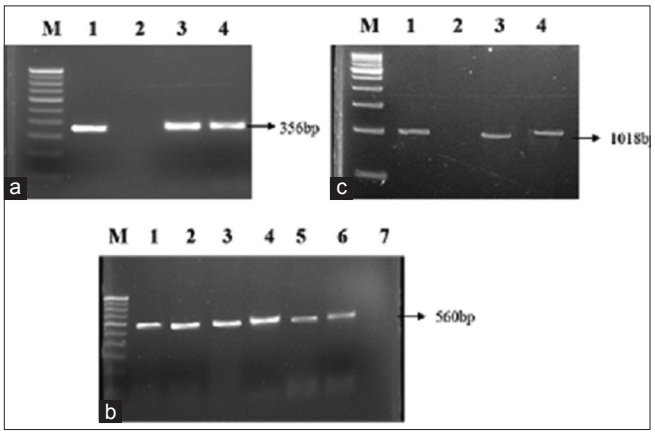

Figure 1: (a) Polymerase chain reaction amplification picture of cefotaximase gene in extended-spectrum beta lactamase producing *Escherichia coli* strains after agarose (1.5%) gel electrophoresis. Lane M: Molecular marker 100 bp DNA ladder; lane 1: Positive control; lane 2: Negative control; lane 3 and 4: *Escherichia coli* clinical isolates. (b) Polymerase chain reaction amplification of temoneira gene. Lane M: 100 bp DNA ladder; lane 1: Positive control; lane 2–6: *Escherichia coli* clinical isolate; lane 7: Negative control. (c) Polymerase chain reaction amplification of sulfhydryl variable gene. Lane M: 500 bp DNA ladder; lane 1: Positive control; lane 2: Negative control; lane 3 and 4: *Escherichia coli* clinical isolates

and imipenem (2%). The data are comparable with a study from Switzerland, in which ESBL-producing *E. coli* urinary isolates showed high resistance rates to amoxicillin/clavulanate, quinolones, and co-trimoxazole and least for nitrofurantoin.[15] Two percent of ESBL *E. coli* showed imipenem resistance which limits the treatment option. It is reported that non-ESBL producing *E. coli* isolates also showed high resistance to antibiotics. In this study, the resistance rate shown by non-ESBL *E. coli* is much higher compared to Kizilca *et al.*'s data, in which non-ESBL producing isolates showed less than 10% resistance rate for nitrofurantoin, fluoroquinolones, and aminoglycosides.[13]

ESBL positivity was significant in male gender (P = 0.034) when compared to females in this study. Colodner *et al.* from Israel also reported male gender as a risk factor associated with community-acquired ESBL-positive UTI.[16] There were no statistical differences between the ESBL- and non-ESBL-producing *E. coli* with regard to age in this study. Previous history of UTI, previous intake of nitrofurantoin and quinolones were identified as risk factors associated with ESBL community-acquired UTI in this study. Other studies from Spain and Turkey have identified previous exposure to antibiotics including third-generation cephalosporins and quinolones and prior UTI history as risk factors associated with community-acquired UTI by ESBL-producing *E. coli*.[1,13]

Regarding molecular characterization of ESBL-producing *E. coli* isolates, high prevalence of CTX-M gene was found in 87.5% of *E. coli* isolates consistent with other studies (Sana *et al.* from Lebanon).[17] In contrast to our results, Rezai *et al.* from Iran observed low prevalence of CTX-M (28%).[18] The frequency of TEM in this study was 68.4% which is comparable to a study by Rezai *et al.* from Iran (49%) and Omar *et al.* from Sudan (55%), respectively.[18,19] The low prevalence of SHV gene (3.1%) reported is similar to the study carried by Sana *et al.* from Lebanon (4%).[17] A study by Jemima *et al.* from Chennai reported SHV genes in 14% and *bla* CTX-M genes in 50% of *E. coli*.[12]

The present study highlights the association between the presence of CTX and TEM gene and resistance to ampicillin and cephalosporin group of antibiotics, which is comparable with the study by Rezai *et al*.[18]

This study was conducted in a single tertiary care center; it may reflect the local demographics and antibiotic resistance patterns and this may be a limitation. In addition, plasmid profile and multilocus sequence typing were not done to measure the DNA sequence variation.

Conclusion

This study demonstrates an increasing trend in the emergence of ESBL *E. coli* in community-acquired

Table 5: Association between antimicrobial resistance and gene expression in extended-spectrum beta-lactamase producing *Escherichia coli*

Antimicrobial agent	CTX		TEM		SHV	
	Absent (*n*=24)	Present (*n*=172)	Absent (*n*=62)	Present (*n*=134)	Absent (*n*=190)	Present (*n*=6)
Amp						
Resistant	11	124*	43	92	131	4
Sensitive	13	48	19	42	59	2
Cfm						
Resistant	21	161	61	121	178	4
Sensitive	3	11	1	13	12	2
CTX						
Resistant	23	165	61	127	183	5
Sensitive	1	7	1	7	7	1
Caz						
Resistant	24	169	62	131	188	5
Sensitive	0	3	0	3	2	1
Cip						
Resistant	13	96	38	71	107	2
Sensitive	11	76	24	63	83	4
Cot						
Resistant	16	97	37	76	111	2
Sensitive	8	75	25	58	79	4
Gen						
Resistant	6	57	23	40	63	0
Sensitive	18	115	39	94	127	6
Lev						
Resistant	4	59	29	34	62	1
Sensitive	20	113	33	100	128	5
Net						
Resistant	2	15	3	14	17	0
Sensitive	22	157	59	120	173	6
Nit						
Resistant	2	28	6	24	30	0
Sensitive	22	144	56	110	160	6
Nx						
Resistant	13	97	39	71	109	1
Sensitive	11	75	23	63	81	5

*Significant differences (P<0.05). Amp = Ampicillin, Cfm = Cefixime, CAZ = Ceftazidime, Cac = Ceftazidime-clavulanic acid, Cip = Ciprofloxacin, Cot = Co-trimoxazole, Do = Doxycycline, Gen = Gentamicin, Ipm = Imipenem, Lev = levofloxacin, Net = Netilmicin, Nit = Nitrofurantoin, Nx = Norfloxacin, CTX = Cefotaximase, TEM = Temoneira, SHV = Sulfhydryl variable

UTI in children in our region warranting the need for ongoing antimicrobial stewardship and surveillance and development of antibiotic guidelines for the management of these complex multidrug-resistant infections. All simple UTIs and complex UTIs should have a culture done and the organisms identified. Overexposure and inappropriate use of antibiotics should be minimized to prevent emergence of ESBL.

It is of great concern that *E. coli* strains carrying *bla* CTX-M and *bla* TEM genes are widespread in Kerala. It is worthwhile that molecular characterization of clinical isolates should be carried out to prevent dissemination of resistant isolates in our community. PCR is a valuable tool for characterization of ESBL producers in clinical and research settings. Determination of CTX-M, TEM, and SHV genes by molecular techniques in ESBL-producing bacteria may give useful data about their epidemiology.

The results emphasize the need for screening ESBL producers and antibiotic profiling along with an assessment of risk factors for managing the children with UTI appropriately to enhance their clinical outcome.

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Conflicts of interest
There are no conflicts of interest.

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