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The detection of ESBL-producing *Escherichia coli* in patients with symptomatic urinary tract infections using different diffusion methods in a rural setting

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Summary
Objectives: This study aimed to determine the prevalence of extended spectrum of beta lactamases (ESBLs), to compare different phenotypic methods for ESBL confirmation and to evaluate the antibiotic resistance patterns among ESBL-producing urinary *Escherichia coli*.
Methods: Urinary *E. coli* isolates that were resistant to at least one of the three indicator cephalosporins (cefotaxime, cefpodoxime and ceftazidime) were tested for ESBL production using the double disc synergy test (DDST), the inhibitory potentiated disc diffusion (IPDD) test and the quantitative E-strip method.
Result: Of the 163 *E. coli* strains isolated, 80 (49%) were resistant to at least one of the three cephalosporins, and 38 (47.5%) tested positive for ESBLs by the IPDD test and the E-strip test. However, only15 (18.7%) strains tested positive by the DDST. Among the third-generation cephalosporins, cefpodoxime (46.1%) was the best screening indicator, followed by ceftazidime (43%) and cefotaxime (39.9%). Most of the ESBL producers (97.3%) were resistant to three or more drugs, compared with 51.2% of non-ESBL producers.
Conclusion: Compared with the DDST, the IPDD and E-strip tests appear to be preferable methods for detecting ESBLs, with better sensitivity (100%) and specificilty (97.6%) and positive predictive values (97.3%). ESBL producers showed significantly

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($p < 0.05$) higher resistance to tobramycin, co-amoxyclav and amikacin than did non-ESBL producers.

Introduction

Despite the widespread availability of antibiotics, urinary tract infections (UTIs) remain the most common bacterial infections in humans [1]. Among the wide array of available antibiotics, β-lactams are the most varied and most widely used agents, accounting for over 50% of all systemic antibiotics in use [2]. The most common cause of bacterial resistance against β-lactam antibiotics is the production of β-lactamases [3]. Many of the second- and third-generation cephalosporins were specifically designed to resist the hydrolytic action of major β-lactamases. However, the evolution of extended-spectrum β-lactamases (ESBLs) has added another weapon to the arsenal of these enzymes. ESBLs are commonly produced by many members of *Enterobacteriaceae*, especially *Escherichia coli* and *Klebsiella pneumoniae*. These organisms efficiently hydrolyze oxyimino-cephalosporins, conferring resistance to third-generation cephalosporins and monobactams [4].

The detection of ESBLs is a challenge for routine clinical microbiology laboratories in resource-limited settings, and the detection of a decrease in susceptibility to oxyimino-cephalosporins is not sufficiently sensitive to detect all ESBL-producing strains. The guidelines developed by the Comite de l'Antibiogramme de la Societe Francaise de Microbiologie [5] (CA-FSM) and the European Committee on Antimicrobial Susceptibility Testing [6] (EUCAST) recommend screening for ESBL-producing isolates based on decreased susceptibility to extended-spectrum cephalosporins in primary antibiotic disc diffusion tests along with one additional confirmatory test. However, the most sensitive method for the phenotypic detection of ESBL remains unknown [7]. Existing phenotypic methods of ESBL detection include disc diffusion-based screening, the double disc synergy test (DDST), inhibitory potentiated disc diffusion (IPDD) and E-strip confirmatory tests.

As per the Clinical and Laboratory Standards Institute (CLSI) guidelines, an initial screen for reduced susceptibility to more than one of the five indicator cephalosporins followed by a confirmatory test can improve the sensitivity of detection. The further identification of specific genes associated with the production of ESBLs can be performed using specific nucleic acid-based assays [8]. However, these molecular assays are limited to resource-rich settings and are beyond the scope of routine microbiology laboratories.

This study aimed to identify a suitable phenotypic method for the detection of ESBL-producing *E. coli* in patients with symptomatic UTI cases in a tertiary care hospital in north-interior Karnataka, which serves a rural population for which data regarding the incidence of ESBL-producing strains is lacking.

Materials and methods

This prospective study was conducted in the Department of Microbiology at Navodaya Medical College Hospital and Research Centre, Raichur, Karnataka, India. A total of 750 consecutive urine samples from patients with symptomatic UTIs were screened. Clean-catch mid-stream urine samples were collected in sterile disposable containers (Uricol, Hi-Media Laboratories Ltd., Mumbai, India) and processed within 1 h. A semi-quantitative loop (Hi-Media Laboratories Ltd., Mumbai, India) measuring 2.2 mm in diameter with a holding capacity of 0.005 mL was employed to culture urine on blood agar and MacConkey agar. The inoculated plates were incubated overnight at 37 °C. Isolates present at a significant number (colony count $\geq 10^5$ CFU/mL) were identified according to standard procedures [9]. Antibiotic susceptibility was tested by the Kirby-Bauer disc diffusion method [10] using antibiotic discs containing ampicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), amikacin (30 μg), imipenem (10 μg), gatifloxacin (5 μg) and tobramycin (10 μg).

Disc susceptibility test to screen for ESBLs

All isolates were screened for ESBL production using three indicator cephalosporins, namely ceftazidime (30 μg), cefotaxime (30 μg) and cefpodoxime (30 μg). The isolates were considered to be resistant if the diameter of the inhibition zone

for ceftazidime, cefotaxime or cefpodoxime was ≤22 mm, ≤27 mm or ≤17 mm, respectively.

The strains that showed resistance to at least one of the three cephalosporins were tested further using phenotypic confirmation methods [10,11].

Double disc synergy test (DDST)

All *E. coli* showing resistance to any of the three indicator cephalosporins were tested for ESBL production by the DDST. Ceftazidime (30 μg), cefotaxime (30 μg), cefpodoxime (30 μg) and amoxicillin/clavulanic acid (amoxicillin 20 μg + clavulanic acid 10 μg) (Hi-Media Laboratories Ltd., Mumbai, India) were used for ESBL detection [12,13]. Amoxicillin/clavulanic acid (20 μg/10 μg) and third-generation cephalosporin discs were placed at a distance of 20 mm from center to center on lawn cultures on Muller—Hinton agar plates. The plates were incubated at 37 °C overnight. Any enhancement in zone of inhibition of cephalosporins towards the amoxicillin/clavulanic acid disc was considered a positive result for an ESBL.

K. pneumoniae ATCC 700603 and *E. coli* ATCC 25922 were used as controls.

Inhibitor potentiated disc diffusion test (IPDD)

The test inoculum was matched to a 0.5 McFarland turbidity standard and streaked onto two Muller—Hinton agar plates, one supplemented with 0.004 mg/L potassium clavulanate (Sigma Aldrich Pvt. Ltd., Bangalore) and another without clavulanate. Ceftazidime (30 μg), cefotaxime (30 μg) and cefpodoxime (30 μg) discs were placed on both of these plates. The agar plates were then incubated at 37 °C overnight. The inhibition zones of the discs were compared between the plates with and without clavulanate. A difference of ≥10 mm in the zone diameter was considered as positive result for an ESBL [14,15].

ESBL E-strip test

The ESBL E-strip test (AB BioMerieux, Solna, Sweden) is based on two gradients; one end of the strip is impregnated with ceftazidime (0.5—32 μg/mL), and the opposite end is impregnated with ceftazidime (0.125—8 μg/mL) and clavulanate (4 μg/mL). The test was performed following the manufacturer's instructions. Briefly, an overnight culture of the test organism on brain heart infusion agar was suspended in saline to match the turbidity to that of a 0.5 McFarland standard. This suspension was then used to inoculate a Muller Hinton agar plate by swabbing the plates with a sterile cotton swab. After drying, the E-test strip was placed on the plate, and the plate was incubated overnight at 37 °C. For both ends of the strip, the point of intersection between the inhibition eclipse and the edge of the E-test strip was considered the MIC. According to the manufacturer, a ceftazidime MIC/ceftazidime-clavulanate MIC ratio ≥8 indicates the presence of ESBL enzymes [8,16].

Statistical analysis

The results were analyzed with descriptive statistics wherever appropriate. The *Chi*-square test was used to evaluate the statistical significance of differences in the results. A p value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS v16.0 software.

Result

A total of 163 *E. coli* strains were isolated in significant numbers from patients with symptomatic UTIs. These strains were subjected to antibiotic susceptibility testing, ESBL screening and phenotype confirmation tests.

Antibiograms revealed that 153 (93.8%) isolates were resistant to ampicillin, 62 (38%) were resistant to trimethoprim/sulfamethoxazole and 61 (37.4%) were resistant to gatifloxacin. Resistance to tobramycin, amoxicillin/clavulanate and amikacin was noted in between 30% and 19.6% of isolates. Only two (1.2%) strains showed resistance to imipenem, as indicated in Table 1.

In a disc-based ESBL screening test, 80 (49%) isolates were resistant to at least one of the three indicator cephalosporins. Resistance was most frequently observed for cefpodoxime ($n = 75$; 46.1%), followed by ceftazidime ($n = 70$; 43%)and cefotaxime ($n = 65$; 39.9%). Among the three cephalosporins, ceftazidime was found to be the best antibiotic for the ESBL phenotypic confirmatory tests when using either the DDST or the IPDD test, as shown in Table 2.

The ESBL E-strip test was considered the gold standard, and the efficacy of the DDST and the IPDD test were analyzed in comparison with the E-strip test. In the DDST, 15 (18.7%) strains were positive for ESBLs, 1 strain gave a false-positive result, and 23 (28.7%) strains gave false-negative results. The IPDD test gave 38 (47.5%) ESBL positives with a ≥10 mm augmentation of the inhibition zone diameter. The mean zone augmentation (95% confidence

Table 1 Comparison of resistance pattern between ESBL positives and negative strains.

Pattern	Resistance pattern ($n = 163$)	ESBL positives strains ($n = 38$)	ESBL negative strains ($n = 125$)	p Value
Ampicillin (A)	153 (93.8%)	38 (100%)	117 (93.6%)	0.19
Amoxyclav (Ac)	38 (23.3%)	14 (36.8%)	24 (19.2%)	0.03
Cotrimoxazole (Co)	62 (38.0%)	20 (52.6%)	42 (33.6%)	0.04
Amikacin (Ak)	32 (19.6%)	13 (34.2%)	19 (15.2%)	<0.02
Imipenem (I)	2 (1.2%)	2 (5.3%)	00	
Gatifloxacin (Gf)	61 (37.4%)	21 (55.3%)	40 (32%)	0.01
Tobramycin (Tb)	49 (30.0%)	16 (42.1%)	33 (26.4%)	0.07
ESBL screening indicators				
Cefpodoxime(CEP)	75 (46.1%)	37 (97.3%)	38 (30.4%)	*<0.0001*
Ceftazidime (CA)	70 (43.0%)	33 (86.8%)	37 (29.6%)	*<0.0001*
Cefotaxime (CE)	65 (39.9%)	35 (92.1%)	30 (29.0%)	*<0.0001*

Table 2 Comparison of screening and confirmatory methods for the detection of extended spectrum β-lactamases.

Sl. no	Cephalosporins	Screening test[a] ($n = 163$)	Confirmatory tests[b] ($n = 80$)		
			DDST	IPDD test	E-strip test
1	Cefpodoxime (CEP)	75	3	14	NA
2	Ceftazidime (CA)	70	7	15	38
3	Cefotaxime (CE)	65	5	09	NA
4	ESBL positives		15	38	38

NA, not applicable.
[a] Disc diffusion test.
[b] Strain showing resistance to at least one cephalosporin indicator antibiotic is selected for confirmatory test.

interval) was 16.6 (13.1, 22.6) mm for ceftazidime, 14.8 (12.4, 18.2) mm for cefotaxime and 19.2 (12.6, 18.4) mm for cefpodoxime, as shown in Table 3.

By the ESBL E-strip test, 38 (47.5%) strains were detected as ESBL producers with a ceftazidime/ceftazidime-clavulanate (TZ/TZL) ratio between 8 and 256. Of the 38 ESBL-positive strains, 34 had a TZ/TZL ratio of between 32 and 256 and a MIC $\log_2$ dilution reduction of $\geq$5. Four strains (5.0%) had a TZ/TZL ratio of 8 with a MIC $\log_2$ dilution reduction of 3—4.5. The remaining 42 (52.5%) strains were ESBL negative, with a ratio of <8 and $\log_2$ reduction of <3,as depicted in Table 4.

Of the 38 ESBL-positive strains, 37 (97.3%) were resistant to cefpodoxime, 35 (92.1%) were resistant to cefotaxime and 33 (86%)were resistant to ceftazidime. Of the 125 ESBL-negative strains, 38 (30.4%) were resistant to cefpodoxime, 37 (29.6%) were resistant to ceftazidime and 30 (29.0%) were resistant to cefotaxime. Significantly more ESBL-positive strains than ESBL-negative strains were resistant to amoxyclav, cotrimoxazole, amikacin and gatifloxacin ($p < 0.05$). Resistance to three or more drugs was significantly more common in ESBL-producing (97.3%) strains than in non-ESBL-producing (51.2%) strains ($p < 0.05$)

Table 3 Phenotypic confirmation ESBLs by E-strip test and their respective $\log_2$ reduction.

No. of strains	Ceftazidime MIC		TZ/TZL ratio	MIC $\log_2$ reduction	%
	Alone (TZ)	With clavulanate (TZL)			
4	0.5	0.38—0.5	1	0	5
5	0.5—2.0	0.25—0.75	2	1	6.2
31	0.5—2.0	0.125—0.75	3—4	2	38.7
6	1.5—2.0	0.19—0.25	6—8	3	7.5
34	4.0—32.0	0.125—0.75	32—256	$\geq$5	42.5

Table 4 Phenotypic confirmation of screening test positive ESBL producers by Inhibitory Potentiated Disc Diffusion test.

Agents	Mean zone diameter ± SD (mm)		Mean zone augmentation (mm) (95% CI)	*p* Value
	MH agar	MH agar + clavulanate		
ESBL positive strains (*n* = 38)				
Ceftazidime	18.6 ± 8.4	39.4 ± 5.2	16.6 (13.1, 22.6)	<0.001
Cefotaxime	22.7 ± 6.6	36.5 ± 4.1	14.8 (12.4, 18.2)	<0.001
Cefpodoxime	16.4 ± 4.9	35.2 ± 4.5	19.8 (12.4, 18.4)	<0.001
ESBL negative strains (*n* = 42)				
Ceftazidime	37.2 ± 3.4	38.1 ± 2.6	1.4 (0.8, 2.4)	<0.001
Cefotaxime	34.5 ± 4.2	36.8 ± 2.1	1.6 (0.5, 2.6)	<0.001
Cefpodoxime	32.3 ± 2.4	35.2 ± 2.2	1.5 (0.6,2.0)	<0.001

CI, confidence interval; MH, Muller Hinton.

Discussion

In the present study, 80 (49%) *E. coli* strains from patients with symptomatic UTIs exhibited resistance to at least one of the three indicator cephalosporins. Of these 80 strains, 38 (23.3%) were identified as ESBL producers by the IPDD and the E-strip tests. However, only15 (18.7%) strains tested positive according to the DDST. Kumar et al. [17] identified 19.8% of *Enterobacteriaceae* isolates as potential ESBL producers by the DDST, with 63.7% of *E. coli* isolates and 14% of *K. pneumoniae* isolates exhibiting ESBL production. In a previous study in Western India, 48.3% of urinary isolates resistant to cefotaxime were ESBL producers [18].

The IPDD test appeared to be a better method than the DDST for confirming ESBL production, showing a sensitivity and specificity of 100% and 97.6%, respectively. The DDST failed to detect 28.7% of ESBL producers, with a sensitivity of 39.4% and a positive predictive value of 93.7%. The sensitivity of the DDST strongly depends on the precise placement of the discs [14]. Previous studies have found that the E-strip test method is sensitive, reliable and convenient [16]; thus, this test was used as the gold standard for identifying ESBL producers in our study.

All cephalosporins used in the IPDD test showed increased inhibition zone diameters (≥10 mm) for ESBL producers. Cefpodoxime had a greater mean zone augmentation (19.2 mm) than ceftazidime (16.6 mm) and cefotaxime (14.8 mm). Our results are in complete agreement with the previous findings of Ho et al. [14] that the IPDD test is more sensitive when ceftazidime is used than when cefotaxime is used. The advantage of the IPDD test is that ESBL producers can be clearly differentiated from non-producers using a cut-off of ≥10 mm for the zone augmentation and three oxyimino-cephalosporins can be tested on one agar plate.

Ceftazidime is considered an excellent substrate for most ESBL enzymes [19,20], and Bush group 2be enzymes can be differentiated from other β-lactamase enzymes (chromosomal or chromosomally derived AmpC enzymes) by the reduction in the ceftazidime MIC in the presence of some clinically useful β-lactamase inhibitors such as clavulanate [21]. Sanders et al. [22] suggested that the Vitek ESBL test and the E-strip test are more sensitive than the disc approximation test for the detection of Bush group 2be enzymes.

One drawback of these phenotypic methods is that inhibitor-resistant β-lactamases might not be detected. The ESBL confirmatory test is based on the demonstration of inhibition by clavulanate. However, other β-lactam resistance factors, including AmpC-type enzymes, porin changes and variants of the original ESBL enzymes, may be co-exist with ESBLs, interfering with the results of these tests.

The need for a two-step strategy (screening and confirmatory tests) due to the presence of a high number of false positives in the initial screening may result in increased turnaround time and costs for the laboratory. Furthermore, the sensitivity of the DDST strongly depends on the precise placement of the discs and the interpretation of the DDST results is more subjective than the interpretation of the E-strip and IPDD results. Thus, IPDD test may be preferred because it is equally sensitive to the E-strip test but less expensive, and a single plate can be used to test more than one cephalosporin.

The antibiotic resistance of ESBL-positive strains was significantly higher ($p < 0.05$) than that of ESBL-negative strains. ESBL producers exhibited resistance to trimethoprim/sulfamethoxazole (52.6%) and amikacin (34.2%). A similar resistance pattern was observed in other studies [23—25]. Resistance

to third-generation cephalosporins was found to co-exist with resistance to fluoroquinolones and aminoglycosides, consistent with the reports of Subha and Ananthan [26] and Dutta Roy et al. [12]. We also observed that most ESBL producers (97.3%) were resistant to two or more drugs, whereas only 51.2% of non-ESBL producers were multidrug resistant. This finding is in agreement with the results of a study by Tankhiwale et al., who reported that multidrug resistance was significantly more common in ESBL producers than in non-producers [18].

In our region, phenotypic data regarding ESBL occurrence are lacking. Further studies including more isolates and using genotypic methods for confirmation are required to validate the above results. In infections with ESBL-producing strains, slight increase in the MICs of oxyimino-cephalosporins has been reported to be sufficient to cause treatment failure. Over reliance on third-generation cephalosporins to treat gram-negative infections is one of the primary factors responsible for the increased resistance to this class of antibiotics, and most of the ESBL-producing strains included in this study were multidrug resistant. The accurate detection and reporting of ESBL production by clinical isolates are therefore crucial. The monitoring and judicious usage of extended-spectrum cephalosporins, periodic surveillance of antibiotic resistance patterns and efforts to decrease empirical antibiotic therapy would be greatly effective in addressing some of the problems associated with ESBLs.

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Conflicts of interest

None.

Ethical approval

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