Indian Journal of Microbiology Research 2021;8(3):243–248

Content available at: https://www.ipinnovative.com/open-access-journals

Indian Journal of Microbiology Research

Journal homepage: https://www.ijmronline.org/

Original Research Article

Evaluation of different phenotypic diffusion methods in the identification of extended spectrum beta lactamase producing uropathogenic *Escherichia coli*

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ARTICLE INFO

Article history:
Received 09-08-2021
Accepted 02-09-2021
Available online 22-09-2021

Keywords:
Uropathogenic E coli
Drug resistance
Extended spectrum beta lactamase 2

ABSTRACT

Objectives: The study was aimed to identify the occurrence of extended spectrum of Beta lactamases (ESBLs), to compare different phenotypic methods used for the confirmation and to evaluate the antibiotic resistance pattern in ESBL producing uropathogenic *Escherichia coli.*

Materials and Methods: The *Escherichia coli* strains were isolated from urine and the isolates resistance to at least one of the three representative cephalosporins (cefotaxime, cefpodoxime and ceftazidime) was tested for ESBL production by Double disc synergy test (DDST), Inhibitory potentiated disc diffusion (IPDD) test and quantitative E-strip method.

Result: Of 120 *Escherichia coli* strains isolated, 62(51.6%) were resistant to at least one of the three cephalosporins and 28 (45.1%) were positive for ESBL by IPDD and E-strip test. However, 9 (14.5%) strains were positive by DDST method. Among third generation cephalosporins, cefpodoxime was (45.8%) better screening indicator followed by ceftazidime (40.0%) and cefotaxime (37.5%). Most of the ESBL producers (97.3%) were resistant to three or more drugs, compared to (51.2%) non-ESBL producers.

Conclusion: The acceptable method for detection of ESBL producing *E.coli* were IPDD and E-strip tests compared to DDST with better sensitivity (100%), specificity (95.8%) and positive predictive value (96.5%). ESBL producers showed significantly ($p<0.05$) higher resistance to tobramycin, amoxyclav and amikacin compared to non ESBL producers.

1. Introduction

In spite of the widespread availability of antibacterial drugs, urinary tract infection (UTI) remains one of the major infections in the community and hospital settings.[1] Amongst the large number of antibiotic drugs, β-lactams are the diverse and largely used antibiotics contributing above 50% of all systemic antimicrobial agents available.[2] The resistance in bacteria towards beta lactam antibiotics is mainly due to the production of beta lactamase enzyme.[3] The second and third generation cephalosporin drugs are precisely designed to neutralize the hydrolytic action of beta lactamase enzymes. Nonetheless, the newest in the reserve of these enzymes by the organisms has been the development of extended spectrum beta lactamases. The ESBL enzymes produced largely by the enteric organisms like, *E. coli* and Klebsiella sps., which hydrolyze oxyimino-cephalosporins leading to the resistance against cephalosporins and monobactams.[4]

The identification of ESBL producing organisms is difficult for routine diagnostic microbiology laboratories of developing countries without molecular diagnostic facilities. Also screening of ESBL producing organisms by monitoring the decrease in susceptibility to oxyimino-

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https://doi.org/10.18231/j.ijmr.2021.050

cephalosporin drugs are not a sensitive tool. The recommended methods for screening ESBL producing organisms are based on the decreased susceptibility to cephalosporins in disc diffusion test.[5] But, the standard protocol for reliable phenotypic method for detection of ESBL is not available.[6] The existing phenotypic methods for ESBL detection are disc diffusion based screening test and double disc synergy test (DDST), Inhibitory potentiated disc diffusion (IPDD) and E-strip confirmatory tests.

According to CLSI guidelines, a screening test for decreased susceptibility to one of the five representative cephalosporin agents, followed by a confirmatory test would increase the chance of identification. Additionally, gene responsible for the production of ESBL enzyme can be detected by molecular methods.[7] But these molecular diagnostic facilities will not normally be available in resource constrained routine microbiology laboratories.

The present research work was intended to find out the appropriate method for the identification of ESBL producing urinary *Escherichia coli*, where the data on the occurrence of ESBL producing *E.coli* is lacking.

2. Materials and Methods

This prospective study was conducted in the Department of Microbiology, Shyam Shah Medical College, Rewa, Madhya Pradesh, India. A total of 500 consecutive urine samples were screened from patients with symptomatic UTI. Clean-catch midstream urine samples were collected in sterile disposable container (Uricol, Hi-Media Laboratories Ltd., Mumbai, India) and processed within one hour. Semi quantitative loop (Hi-Media Laboratories Ltd., Mumbai, India) measuring 2.2 mm diameter with a holding capacity of 0.005 ml was employed to culture urine on CLED agar and MacConkey's agar. The inoculated plates were incubated over night at 37^oC. Isolates in significant number (colony count $\geq 10^5$ CFU/ml) were identified by standard procedures.[8] Antibiotic susceptibility test was done by Kirby-Bauer disc diffusion method[9] using antibiotic discs: ampicillin (10μg), amoxicillin/clavulanic acid (20/10μg), co-trimoxazole (1.25/23.75 μg), amikacin (30μg), imipenem (10μg), gatifloxacin (5μg) and tobramycin (10 μg).

2.1. Disc susceptibility test to screen ESBLs

All the isolates were screened for ESBL production by using three indicator cephalosporins, namely ceftazidime (30 μg), cefotaxime (30 μg) and cefpodoxime (30 μg). The isolates were considered to be resistant, if the inhibition zone diameter of ceftazidime, cefotaxime and cefpodoxime were < 22mm, <27mm and <17mm respectively.

The strains which showed resistance to at least one of the three cephalosporins was further included for phenotypic confirmation method.[9,10]

2.2. Double disc synergy test (DDST)

The *Escherichia coli* showing decreased susceptibility to any of the three cephalosporins used were further tested for ESBL production by DDST method. Ceftazidime, cefotaxime, cefpodoxime and amoxy-clav (Hi-Media Laboratories Ltd., Mumbai, India) were used in this method.[11,12] Over the lawn cultured Muller-Hinton agar plates, amoxy-clav and third generation cephalosporin discs were placed at a distance of 20mm from the center. The Plates were incubated at 37ºC for 8 hours. The augmentation in the zone of inhibition of cephalosporins towards the amoxy-clav disc was considered to be positive for ESBL. The standard strains of Klebsiella pneumoniae ATCC 700603 and *E. coli* ATCC 25922 were used as controls.

2.3. Inhibitor potentiated disc diffusion test (IPDD)

The turbidity of *E.coli* in a broth was matched with 0.5 McFarland turbidity standards and inoculated onto two Muller-Hinton agar plates by streak method. Of the two plates, one was supplemented with 0.004 mg/L Potassium clavulanate (Sigma Aldrich Pvt Ltd, Bengaluru) and another without clavulanate. The ceftazidime, cefotaxime and cefpodoxime disks were placed on both of these plates. The inoculated agar plates were then incubated at 37ºC for 8 hours. The inhibition zones of disks were compared between the plates with and without potassium clavulanate. The difference in the zone size of $\geq$ 10 mm diameter was taken as positive for the production of ESBL.[13,14]

2.4. ESBL Epsilometer-strip test (E-strip test)

The commercially available ESBL E-strip (make: AB Biomerieux) contains two gradients of antibiotic drugs. At one end, the strip is impregnated with ceftazidime (0.5 to 32 mg/ml) and on the other end is with ceftazidime (0.125 to 8 mg/ml) with clavulanate (4 mg/ml). The overnight growth of *E.coli* isolate was suspended in saline to match the turbidity with 0.5 McFarland standards and was then inoculated on Muller Hinton agar plate by lawn culture technique. After drying, the E -test strip was placed on the plate and incubated overnight at 37ºC. The MICs on both ends of the E- strip were interpreted as the point of intersection of the inhibition eclipse with the E-test strip edge. The ratio of ceftazidime/ ceftazidime with clavulanate MIC $\geq$ 8 indicates the presence of ESBL enzymes.[7,15]

2.4.1. Statistical analysis

The results of the study were statistically analyzed using SPSS v 16.0 software wherever suitable. The Chi- square test was done to analyze statistical significance. The p-value of less than 0.05 was considered statistically significant.

3. Result

In our study, 120 symptomatic urinary tract infection cases were diagnosed as significant bacteriuria due to *Escherichia coli* by calibrated loop culture technique. The isolated *E. coli* strains were further used for antibiotic susceptibility testing and ESBL detection.

The antibiogram results shown that 110 (91.6%) *E.coli* isolates were resistant to ampicillin followed by 42 (35.0%) isolates to co-trimoxazole and 41 (34.1%) isolates to gatifloxacin. However, resistance to tobramycin, amoxyclav and amikacin were recorded as 31.6%, 19.1% and 15.0% respectively. Only one (0.8%) strain has shown resistance to imipenem as depicted in the Table 1.

In the DDST and IPDD screening test for ESBL production, 62 (51.6%) isolates were resistant to at least one of the three representative cephalosporin drugs. The highest resistance was observed with cefpodoxime (n=55; 45.8%) followed by ceftazidime (n=48; 40%) and cefotaxime (n=45; 37.5%). Out of the three cephalosporins tested in the study, ceftazidime was found to be the better antibiotic drug for the identification of ESBL production by both DDST and IPDD (Table 2).

In the present study, the efficacy of DDST and IPDD disc diffusion tests were compared with ESBL E-strip test. By DDST method, 9 (14.5%) strains were positive for ESBL, one strain was false positive and 18 (29.0%) strains showed false negative result. The IPDD test showed 28 (45.1%) as mentioned in Table 3.

The ESBL positives with augmentation zone of inhibition diameter is ≥ 10mm.The mean zone augmentation (95% CI) was 16.2 (12.8, 21.4) mm for ceftazidime, 13.9 (12.2, 18.0) mm for cefotaxime and 18.6 (12.2, 18.6) mm for cefpodoxime as mentioned in Table 4.

The ESBL E-strip test results showed, 28 (45.1%) *E.coli* isolates were identified as ESBL producers with Ceftazidime/ceftazidime-clavulanate (TZ/TZL) ratio between 8 and 256. Of 28 ESBL positives, 26 isolates showed TZ/TZL ratio of between 32 and 256 with MIC $\log_2$ dilution reduction ≥ 5. The remaining 34 (54.8%) *E.coli* isolates were negative for ESBL production with the ratio less than 8 and $\log_2$ reduction less than 3 (Table 4).

In 28 ESBL positives *E.coli*, 27 (96.4%) isolates have shown resistance to cefpodoxime followed by 25 (89.2%) strains to cefotaxime and 23 (82.1%) strains to ceftazidime. Out of 92 Non-ESBL isolates, 28 (30.4%) were resistant to cefpodoxime followed by 25 (27.1%) strains toceftazidime and 20 (21.7%) strains to cefotaxime. The ESBL positive strains exhibited statistically significant ($p<0.05$) resistance to tobramycin followed by amoxyclav and amikacin compared to non-ESBL isolates. The resistance to multi drugs was noticeable in ESBL producing (96.7%) isolates compared to non- ESBL producing (52.1%) isolates, which was statistically significant ($p<0.05$).

4. Discussion

In the our study, 62 (51.6%) *Escherichia coli* strains from UTI cases showed resistant to one of the three representative cephalosporin drugs. Out of these 62 *E.coli* isolates, 28 (45.1%) were found ESBL producers by IPDD test and E-strip test. However, only 9 (14.5%) strains were positive by DDST method. A study from Hyderabad (TS) reported that, 19.8% Enterobacteriaceae were potential ESBL producers by double disc synergy test with 63.7% of *Escherichia coli* and 14% of Klebsiella pneumoniae shown ESBL production.[16] In another study from Western part India,48.3% ESBL producing urinary isolates were resistant to cefotaxime drug.[17]

In comparison to DDST, IPDD test appeared to be better methods for confirming ESBLs shown a sensitivity of 100% and specificity of 95.8%. The DDST method was unsuccessful to detect 30.6% ESBL producing isolates with a low sensitivity of 42.2% and positive predictive value of 91.6%. The sensitivity of the DDST test strongly relies on the accurate location of discs on the culture plate.[13] The earlier studies have shown that the ESBL E-strip test was comparatively more sensitive, dependable and appropriate method,[15] based on which it was used as the gold standard test for confirming ESBL phenotypically in the present study.

The three representative cephalosporin drugs used in IPDD test showed increased zone of inhibition (≥10mm) to ESBL producers. The cefpodoxime showed superior mean zone augmentation (18.6 mm) compared to mean zone augmentation of ceftazidime (16.2 mm) and cefotaxime (13.9 mm). In our study, the IPDD test was more sensitive with ceftazidime than cefotaxime, which is comparable with the previous work by Ho et al.[13] The benefit of IPDD test is that the ESBLs could be easily separated from non-ESBL producing organisms by a break point of ≥ 10 mm zone augmentation and more than one cephalosporin drug can be tested on single test plate.

The Ceftazidime is identified as an exceptional substrate for most ESBL enzymes[18,19] and Bush group 2be enzymes can be differentiated from other beta lactamase enzymes by the decrease in ceftazidime MIC in presence of beta lactamase inhibitors like clavulanate.[20] Another study suggested that the automated technique like Vitek and Epsilometer–strip tests are sensitive and reliable compared to the disc diffusion tests.[21]

The disadvantage of these diffusion methods is that, they may not detect inhibitor-resistant beta lactamases. The ESBL confirmatory test is based on the demonstration of inhibition by clavulanate. But, other mechanisms of beta lactam resistance, like AmpC enzymes, change in the porin channel and variants ESBL enzymes may be present or co-exist with ESBL, which interfere in the results of these diffusion tests.

Table 1: Association between drug resistance pattern and ESBL producing *E.coli* strains

Pattern	Resistance pattern (n=120)	ESBL positives strains (n=28)	ESBL negative strains (n=92)	- Value
Ampicillin (A)	110 (91.6%)	28 (100%)	82 (89.1%)	0.19
Amoxyclav (AC)	23 (19.1%)	10 (35.7%)	13 (14.1%)	0.012
Cotrimoxazole (Co)	42 (35.0%)	15 (53.5%)	27 (29.3%)	0.0099
Amikacin (AK)	18 (15.0%)	9 (32.1%)	9 (9.7%)	<0.0001
Imipenem (I)	1 (0.8%)	1 (3.5%)	00	
Gatifloxacin (GF)	41 (34.1%)	15 (53.5%)	26 (28.2%)	0.12
Tobramycin (Tb)	38 (31.6%)	13 (46.4%)	25 (27.1%)	0.0003
ESBL screening indicators:				
Cefpodoxime(CEP)	55 (45.8%)	27 (96.4%)	28 (30.4%)	<0.0001
Ceftazidime (CA)	48 (40.0%)	23 (82.1%)	25 (27.1%)	<0.0001
Cefotaxime (CE)	45 (37.5%)	25 (89.2%)	20 (21.7%)	<0.0001

Table 2: Comparisonof different diffusion methods for the detection of extended spectrum of beta lactamases

S.No	Cepahlosporins	Screening test* (n=120)	Confirmatory tests** (n=62)		
			DDST	IPDD test	E-strip test
1	Cefpodoxime (CEP)	55	1	11	NA
2	Ceftazidime (CA)	48	5	12	28
3	Cefotaxime (CE)	45	3	05	NA
4	ESBL positives		9	28	28

* Disc diffusion test
**The *E.coli* strain showing resistance to at least one cephalosprin indicator antibiotic is selected for confirmatory test.
NA – Not Applicable

Table 3: Detection of ESBL producing *E.coli* strains 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)			
2	0.5	0.38-0.5	1	0	3.2
3	0.5-2.0	0.25-0.75	2	1	4.8
24	0.5-2.0	0.125-0.75	3-4	2	38.7
2	1.5-2.0	0.19-0.25	6-8	3	3.2
26	4.0-32.0	0.125-0.75	32-256	≥5	41.9

Table 4: Confirmation of screening test positive ESBL producers by inhibitory potentiated disc diffusion (IPDD) test

Agents	Mean Zone diameter± S.D (mm)		Mean Zone augmentation (mm) (95% CI)	-Value
	MH Agar	MH agar + Clavulanate		
ESBL Positive strains(n=28)				
Ceftazidime	18.4±8.2	38.2±4.8	16.2 (12.8, 21.4)	<0.001
Cefotaxime	21.6±6.4	34.2 ±3.9	13.9 (12.2, 18.0)	<0.001
Cefpodoxime	16.2±4.7	34.6±4.2	18.6 (12.2, 18.6)	<0.001
ESBL Negative strains(n=34)				
Ceftazidime	36.8±3.2	36.6±2.2	1.6 (0.8, 2.4)	<0.001
Cefotaxime	32.8±4.6	34.8±2.2	1.4 (0.4, 2.6)	<0.001
Cefpodoxime	32.2±2.4	35.4 ±2.6	1.6 (0.6,2.0)	<0.001

CI, Confidence interval
MH– Muller Hinton

Due to the presence of a large amount of false positives in the screening procedures, the two steps strategy (screening and confirmatory tests) may be adapted. Even the sensitivity of DDST depends on the accurate placement of the discs on the agar plate and interpretation of DDST results is more subjective compared to recording the results of E-strip test and IPDD test. Therefore, IPDD test may be preferred over E-strip test, as it is equally sensitive, cost effective and more than one cephalosporin drug can be used per test.

The drug resistance of ESBL producing *E.coli* were significantly higher ($p < 0.05$) than the non-ESBL producing isolates. The ESBL producing isolates showed greater resistance to co-trimoxazole (53.5%) and amikacin (32.1%), which is comparable with the study done by Spanu et al., Baby Padmini et al. and Menon et al.[22–24] The cephalosporin resistant organisms have shown resistance to other antibiotics classes like fluoroquinolones and aminoglycosides which are in concordance with the other study reports.[10,25] It was also noticed that, most of the ESBL producing *E.coli* (96.7%) were resistant to two or more drugs compared to Non-ESBL producing isolates (52.1%). This finding was in complete agreement with a study conducted by Tankhiwale et al., as they also reported the significantly higher multidrug resistancein ESBL producing isolates than in non ESBL producers.[17]

In our area, the records pertaining to the incidence of ESBL producing isolates is very limited. In addition, confirmatory molecular methods need to be carried out in the identification of ESBL producing isolates to validate the results of different phenotypic diffusion methods. Probably too much reliance and extensive use of third generation cephalosporin drugs in the treatment of enteric gram negative organisms has been the principal factor responsible for increased drug resistance to cephalosporins and other class of antibiotics. The precise identification of ESBL producing isolates, judicious use of broad spectrum antibiotics, periodic surveillance of antibiotic resistance pattern and efforts to decrease empirical antibiotic therapy would go a long way in addressing some of the issues related with ESBL production in clinical isolates.

5. Source of Funding

None.

6. Conflict of Interest

None.

Acknowledgment

We thank the faculty of Dept. of Microbiology Navodaya Medical College, Raichur and M R Medical College, Kalaburagi for their constant inspiration in conducting this work.

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Cite this article: Nigudgi A, Hajare V, Biradar S, Anandkumar H. Evaluation of different phenotypic diffusion methods in the identification of extended spectrum beta lactamase producing uropathogenic *Escherichia coli*. *Indian J Microbiol Res* 2021;8(3):243-248.