

Phenotypic detection of extended spectrum β -lactamase and Amp-C β -lactamase producing clinical isolates in a Tertiary Care Hospital: A preliminary study

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Abstract

Background: Production of β -lactamase enzymes by Gram-negative bacteria is the most common mechanism to acquire drug resistance to β -lactam antibiotics. Limitations in detecting extended spectrum β -lactamases (ESBL) and Amp-C β -lactamases have contributed to the uncontrolled spread of bacterial resistance and are of significant clinical concern. **Materials and Methods:** A total of 148 samples was selected on the basis of resistance against third-generation cephalosporin for screening ESBLs and Amp-C β -lactamases production. These multidrug-resistant strains were phenotypically screened for ESBL production by phenotypic confirmatory disc diffusion test and double disc synergy test. Modified three-dimensional method was used for Amp-C β -lactamases detection. **Result:** Among the 148 isolates, 82 (55.40%) were ESBL producers, and 115 (77.70%) were Amp-C β -lactamases producers. Co-existence of ESBL and Amp-C was observed in 70 (47.29%) isolates. *Escherichia coli* was the most common ESBL and Amp-C β -lactamase producer. All ESBL producers were highly resistant to ciprofloxacin (83.10%), cotrimoxazole (95.27%), and gentamicin (89.18%). However, these bacterial strains were sensitive to imipenem 146 (98.64%) and piperacillin/tazobactam 143 (96.62%). **Conclusion:** Our study showed that ESBL producing organisms were not only resistant to cephalosporins but also to other group of drugs and also that multiple mechanisms play a role in drug resistance among Gram-negative bacteria.

Key words: Amp-C β -lactamase, extended spectrum β -lactamase, Gram-negative bacteria, minimum inhibitory concentration, multidrug resistance

INTRODUCTION

β -lactamase enzymes production is the most common mechanism of developing drug resistance to β -lactam antibiotics among Gram-negative bacteria.^[1] Extended spectrum β -lactamase (ESBLs) and Amp-C β -lactamase

mediated resistance are of increasing clinical concern.^[2] ESBLs are group of enzymes which confers resistance to extended spectrum cephalosporins, aztreonam, and oxyimino β -lactams and are inhibited by β -lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam.^[3] These enzymes are commonly found in Enterobacteriaceae and other Gram-negative organisms such as *Pseudomonas aeruginosa*^[4] and are encoded by mutated TEM1, TEM2 and SHV genes on plasmids.^[5] Amp-C class of β -lactamase are cephalosporinase, belong to the molecular class 'C' of Ambler's classification^[6] and are both plasmid and chromosomal mediated and are not inhibited by β -lactamase inhibitors. They hydrolyze the cephamycins and carbapenems are the only antibiotic

Access this article online	
Quick Response Code:	Website: www.jnsbm.org
	DOI: 10.4103/0976-9668.160014

effective against them.^[7] The spread of these ESBLs and Amp-C β -lactamase producing strains limits the use of β -lactam class of antibiotic, causing serious therapeutic failures, compelling use of more broad spectrum and expensive drugs.^[3] The present study was designed to know the prevalence of ESBL and Amp-C β -lactamase producers among Gram-negative organisms in our hospital.

MATERIALS AND METHODS

A prospective cross-sectional study, following approval by the institution ethics committee was conducted over a period of 1-year in a tertiary care hospital in Puducherry, India. A total of 148 nonrepetitive Gram-negative bacilli, resistant to one or more third generation cephalosporin (3GC) isolated from various clinical samples like urine (69), pus (68), sputum (5), blood (4), body fluids (2) were tested for ESBL and Amp-C production. The organisms isolated were identified using standard methods. The antibiotic sensitivity testing was performed by Kirby-Bauer disc diffusion method to the following antibiotics ampicillin (30 μ g), amoxicillin-clavulanic acid (20 μ g/10 μ g), gentamicin (10 μ g), amikacin (30 μ g), trimethoprim/sulphamethoxazole (1.25/23.75 μ g), ciprofloxacin (5 μ g), ceftazidime (30 μ g), ceftriaxone (30 μ g), cefotaxime (30 μ g), piperacillin (100 μ g), piperacillin-tazobactam (100 μ g/10 μ g), and imipenem (10 μ g). The results were interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines.^[8] The isolates were screened for ESBLs and Amp-C β -lactamases production by phenotypic method. *Klebsiella pneumoniae* ATCC 700603 was used as a positive control and *Escherichia coli* ATCC 25922 as a negative control.

Screening for extended spectrum β -lactamase

Phenotypic confirmatory disc diffusion test

Mueller Hinton agar (MHA) was inoculated with standard inoculum (0.5 McFarland) of the test isolate. It was tested for ceftazidime (30 μ g) and ceftazidime - clavulanic acid (30 μ g/10 μ g). An increase in zone diameter of ≥ 5 mm in the presence of clavulanic acid than ceftazidime alone was interpreted as ESBL producer [Figure 1].^[6,8]

Double disc synergy test

Mueller Hinton agar was inoculated with the standard (0.5 McFarland) inoculum of the test isolate. Ceftazidime (30 μ g) disc was placed on agar 15 mm away from the center of amoxicillin-clavulanic acid (20 μ g/10 μ g) disc. Extension of zone of inhibition towards amoxicillin-clavulanic acid was interpreted as ESBL producer [Figure 2].^[6,8]

Minimum inhibitory concentration determination

Minimum inhibitory concentration (MIC) to ceftazidime was determined by agar dilution method for the ESBL producing strains using an inoculum size of 10^5 cfu/ml. MHA plates were prepared by incorporating two-fold dilution of antibiotic and the range tested was 0.1-1024 μ g/ml. 10 μ l of the test isolate was inoculated on the plates with different dilutions of the antibiotic. MIC of ≥ 2 μ g/ml was regarded as possible ESBL producer.^[4,9]

Screening for plasmid Amp-C β -lactamase

Modified three-dimensional method

About 10-15 g of bacterial overnight growth from MHA was transferred to pre-weighed sterile microcentrifuge tube; it was suspended in peptone water and centrifuged at 3000 rpm for 15 min. Crude enzyme was extracted by repeated freezing and thawing of the bacterial pellet (10 cycles). Lawn culture of *E. coli* ATCC 25922 was prepared on MHA plate and cefoxitin disc was placed on a plate, a linear slit of 3 cm was made using sterile surgical blade, 3 mm away from cefoxitin disc. At the other end of the slit, a small circular well was made and about 30-40 μ l of the enzyme extract was loaded into the well. It was then kept upright for 5-10 min for the liquid to dry and incubated at 37°C. An indentation in the zone of inhibition at the point where the slit is made was considered positive for three-dimensional test [Figure 3].^[2]

RESULTS

A total of 403 Gram-negative pathogens was isolated from various clinical samples. Among these Gram-negative pathogens, 148 (36.72%) were resistant to one or more 3GC and were screened for ESBL and Amp-C β -lactamase production by phenotypic method. 82

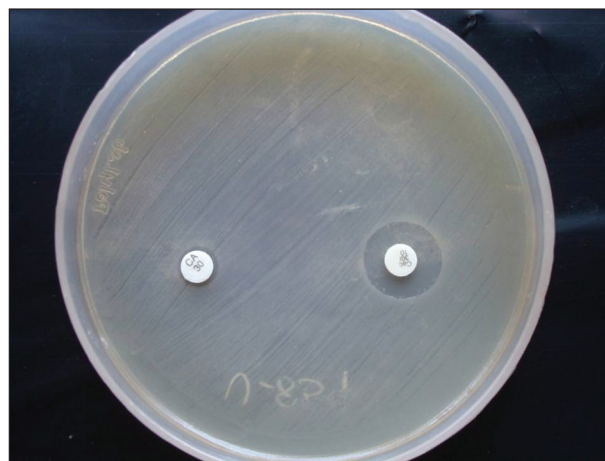


Figure 1: Phenotypic confirmatory disc diffusion test — showing an increase in zone size of >5 mm for ceftazidime-clavulanic acid



Figure 2: Double disc synergy test — showing an enhancement of zone towards amoxicillin-clavulanic acid

(55.40%) ESBL producing organisms were identified. Among them, 79 (53.37%) were positive by phenotypic confirmatory disc diffusion test (PCDDT) and 68 (45.94%) by double-disc synergy test (DDST). The most common ESBL producing organisms were *E. coli* 60 (77.02%) followed by *Klebsiella spp.* 13 (16.4%) and *Pseudomonas spp.* 3 (3.79%) [Table 1]. MIC of the isolates resistant to 3GC was in the range of 16-512 $\mu\text{g}/\text{ml}$ for ceftazidime [Table 2]. Amp-C β -lactamase production was observed in 115 (77.70%) isolates. The most common Amp-C β -lactamase producer was *E. coli* 69 (46.62%) followed by *Klebsiella spp.* 19 (12.83%) and *Pseudomonas spp.* 3 (2.02%). Co-existence of both ESBL and Amp-C β -lactamase was observed in 70 (47.29%) isolates [Table 1]. All the ESBL and Amp-C producers were sensitive to imipenem 146 (98.64), piperacillin\tazobactam 143 (96.62) and amikacin 78 (52.70%). They were highly resistant to gentamicin (89.18%), cotrimoxazole (95.27%), and ciprofloxacin (83.10%) [Table 3].

DISCUSSION

Emergence of ESBLs and Amp-C β -lactamase producing Gram-negative organisms presents significant diagnostic and therapeutic challenge in the management of infection.^[10] The risk factor for the colonization or infection with these organisms is due to prolonged hospital stay, intensive care unit admission, urinary and arterial catheterization and exposure to antibiotics including extended spectrum cephalosporins.^[4] Detection of these resistant isolates is difficult based on routine susceptibility testing performed by clinical microbiology laboratory. CLSI provides recommendations for testing for ESBLs among Gram-negative organisms, but there are no CLSI recommended tests for Amp-C detection. Various phenotypic methods



Figure 3: Modified three-dimensional method for Amp-C beta lactamase — showing indentation of zone for positive isolates (arrow) and no indentation for negative control (ATCC *Escherichia coli* 25922)

have been described to detect Amp-C β -lactamase. Among these, the three-dimensional test is considered as the gold standard for Amp-C detection, but it is labor intensive.^[11]

The incidence of ESBL in various studies reported in India varies from 60% to 80%.^[10] In the present study, out of 148 Gram-negative organisms resistant to 3GC, ESBL production was observed in 82 (55.40%) isolates, a prevalence rate consistent with other reports.^[2,10] PCDDT was more sensitive than DDST for detection of ESBL.^[11]

Klebsiella spp. and *E. coli* with MIC ≥ 2 $\mu\text{g}/\text{ml}$ against ceftazidime, ceftazidime, aztreonam, cefotaxime, and ceftriaxone should be regarded as possible ESBL producer (CLSI guidelines).^[8,12] In the present study, the MIC of the isolates that were resistant to 3GC were in the range of 16-512 $\mu\text{g}/\text{ml}$ against the ceftazidime. 70.3% of the isolates had an MIC of 256 $\mu\text{g}/\text{ml}$, and these clinical isolates were from the pyogenic infection, but the MIC of the urinary isolates was variable.

Various methods for Amp-C β -lactamase detection are previously described. In our study, we have used modified three-dimensional test for Amp-C β -lactamase detection, with 115 (77.70%) isolates being positive, which is significantly higher than previous reports.^[2,5,13] Amp-C β -lactamase production was more common among *E. coli* 69 (46.62%) in our study. Amp-C β -lactamase when present along with the ESBL will mask the phenotype of the latter.^[11] We observed co-existence of ESBL and Amp-C β -lactamase in 47.29% of the isolates, which is consistent with previous reports.^[2,7,11] The β -lactamase producing organisms shows resistance not only to extended spectrum cephalosporin but

Table 1: Number of ESBL and Amp-C β -lactamase producers among Gram-negative organisms-*n* (%)

Organisms	No. of isolates resistant to 3GC	PCDDT (ESBL) (%)	DDST (ESBL) (%)	Amp-C producers (%)	Both ESBL and Amp-C producers (%)
<i>E. coli</i>	91	57 (77.02)	50 (73.52)	69 (46.62)	53 (35.81)
<i>Klebsiella spp.</i>	23	12 (6.16)	9 (13.23)	19 (12.83)	10 (6.75)
<i>Pseudomonas spp.</i>	14	2 (2.70)	3 (4.41)	11 (7.43)	3 (2.02)
<i>Proteus spp.</i>	10	3 (4.05)	3 (4.41)	8 (5.40)	3 (2.02)
<i>Acinetobacter spp.</i>	5	1 (1.35)	0	4 (2.70)	0
<i>Citrobacter spp.</i>	3	2 (2.70)	2 (2.94)	3 (2.02)	2 (2.02)
<i>Enterobacter spp.</i>	1	1 (1.35)	0	0	0
<i>S. flexneri</i>	1	1 (1.35)	1 (1.47)	1 (0.67)	1 (0.67)
Total	148	79 (53.37)	68 (45.94)	115 (77.70)	70 (47.29)

ESBL: Extended spectrum β -lactamases, *E. coli*: *Escherichia coli*, *S. flexneri*: *Shigella flexneri*, PCDDT: Phenotypic confirmatory disc diffusion test, DDST: Double disc synergy test

Table 2: MIC of ceftazidime of the isolates resistant to 3GCs – *n* (%)

Clinical isolates	Number of isolates resistant to 3GC (%)	MIC in μ g/ml					
		16 (%)	32 (%)	64 (%)	128 (%)	256 (%)	512 (%)
<i>E. coli</i>	91	7 (7.6)	13 (14.2)	9 (9.8)	11 (12.1)	49 (53.8)	2 (2.1)
<i>Klebsiella spp.</i>	23	0	0	0	0	22 (95.6)	1 (4.3)
<i>Pseudomonas spp.</i>	14	0	0	0	0	14 (100)	0
<i>Proteus spp.</i>	10	0	0	0	0	10 (100)	0
<i>Acinetobacter spp.</i>	5	0	0	0	0	4 (80)	1 (20)
<i>Citrobacter spp.</i>	3	0	0	0	0	3 (100)	0
<i>Enterobacter spp.</i>	1	0	0	0	0	1 (100)	0
<i>S. flexneri</i>	1	0	0	0	0	1 (100)	0
Total	148	7 (4.7)	13 (8.7)	9 (6.1)	11 (7.4)	104 (70.3)	2 (1.3)

MIC: Minimum inhibitory concentration, *E. coli*: *Escherichia coli*, 3GC: Third generation cephalosporin, *S. flexneri*: *Shigella flexneri*

Table 3: Antibiotic sensitivity pattern of ESBL and Amp-C β -lactamase producing organisms – *n* (%)

Antibiotic	<i>E. coli</i> (91)	<i>Klebsiella sp</i> (23)	<i>Pseudomonas sp</i> (14)	<i>Proteus sp</i> (10)	<i>Acinetobacter sp</i> (5)	<i>Citrobacter sp</i> (3)	<i>Enterobacter sp</i> (1)	<i>S. flexneri</i> (1)
Ampicillin	0	0	0	0	0	0	0	0
Amoxicillin-clavulanic acid	0	0	0	0	0	0	0	0
Gentamicin	7 (7.69)	4 (17.39)	0	1 (9)	2 (40)	0	1 (100)	0
Amikacin	64 (70.32)	9 (39.13)	1 (7.14)	1	2 (40)	0	1 (100)	0
Co-trimoxazole	4 (4.39)	1 (4.34)	0	0	1 (20)	0	0	0
Ciprofloxacin	2 (2.19)	4 (17.39)	3 (21.32)	3 (27.27)	1 (20)	1 (33.33)	1 (100)	0
Ceftazidime	0	0	0	0	0	0	0	0
Ceftriaxone	0	0	0	0	1 (20)	0	0	0
Cefotaxime	0	0	0	0	0	0	0	0
Piperacillin	0	0	0	0	0	0	0	0
Piperacillin-tazobactam	90 (98.90)	22 (95.65)	13 (92.85)	9 (90.90)	5 (100)	1 (33.3)	1 (100)	1 (100)
Imipenem	91 (100)	23 (100)	14 (100)	10 (100)	3 (60)	1 (33.3)	1 (100)	1 (100)

ESBL: Extended spectrum β -lactamases, *E. coli*: *Escherichia coli*, *S. flexneri*: *Shigella flexneri*

also to other antimicrobials such as aminoglycosides, sulfonamides, and fluoroquinolones.^[14] Carbapenems and piperacillin/tazobactam are the most active drug in the treatment of these infections.^[15] In our study, all the ESBL and Amp-C producer were highly resistant to gentamicin, cotrimoxazole, and ciprofloxacin. However, they were sensitive to imipenem (98.64%), piperacillin/tazobactam (96.62%), and amikacin (52.70%). Even though the studies have shown that the ESBL and Amp-C producers were sensitive to imipenem, amikacin, and ciprofloxacin,^[7] a study from South India reported a resistance of 3% to imipenem.^[13]

CONCLUSION

The prevalence of ESBL and Amp-C production varies periodically in different regions, which limits the clinical use of β -lactams. Early detection of ESBL and Amp-C β -lactamase is of paramount importance for surveillance and control of antibiotic resistance and must be routinely evaluated in all hospital settings. Our study identifies the multiple mechanisms involved in drug resistance among Gram-negative bacteria and supports use of genotypic method to detect types of ESBL and Amp-C β -lactamase production.

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How to cite this article: Sageerabanoo S, Malini A, Mangaiyarkarasi T, Hemalatha G. Phenotypic detection of extended spectrum β -lactamase and Amp-C β -lactamase producing clinical isolates in a Tertiary Care Hospital: A preliminary study. *J Nat Sc Biol Med* 2015;6:383-7.

Source of Support: Nil. **Conflict of Interest:** None declared.