PREVALENCE OF EXTENDED SPECTRUM BETA-LACTAMASES AND AMP-C BETA-LACTAMASES IN CLINICAL ISOLATES OF GRAM-NEGATIVE BACILLI AT A TERTIARY CARE HOSPITAL

Malini Jagannatha Rao¹, Shruthi Harle², Padmavathy M³

¹Specialist cum Assistant Professor, Department of Microbiology, ESIC-MC-PGIMSR, Bangalore, Karnataka, India.

²Specialist, Department of Microbiology, ESIC-MC-PGIMSR, Bangalore, Karnataka, India.

³Specialist cum Assistant Professor, Department of Microbiology, ESIC-MC-PGIMSR, Bangalore, Karnataka, India.

ABSTRACT

BACKGROUND

Amp-C beta-lactamase are cephalosporinases that hydrolyze cephamycins as well as other extended spectrum cephalosporins and are poorly inhibited by clavulanic acid. Although, reported with increasing frequency, the true rate of occurrence of Amp-C β -lactamases in different organisms including members of Enterobacteriaceae remains unknown.

The present study was designed to determine the occurrence of Extended Spectrum Beta-Lactamases (ESBL) and Amp-C beta-lactamase enzyme harbouring Gram-negative clinical isolates in a tertiary care hospital in South India.

MATERIALS AND METHODS

A total of 205 Gram-negative bacteria were screened for ESBL and Amp-C beta-lactamases. Isolates found to be positive by the screening test were subjected to the confirmatory test for ESBL and Amp-C disk test for plasmid Amp-C production.

RESULTS

Of the 205 clinical isolates, 135 (65.9%) were screen test positive for ESBL. Confirmatory test for ESBL revealed 79 (38.5%) isolates to be positive and 71 isolates were screen test positive for Amp-C beta-lactamase. Plasmid Amp-C was detected in 32 (15.6%) by Amp-C disc test. Co-production of ESBL and Amp-C was seen in 9 (4.4%) isolates and maximum coexistence was seen in *Klebsiella pneumoniae* (15.3%) isolates.

CONCLUSION

Screening and confirmatory tests are necessary to identify ESBL and true Amp-C producers, as it is a simple and rapid procedure. The high incidence of beta-lactamase production due to multiple mechanisms is alarming and requires urgent action from both a therapeutic and infection control perspective, so that proper antibiotic policy and appropriate measures could be taken to restrict the indiscriminate use of cephalosporins and carbapenems, so as to minimise the emergence of this multiple beta-lactamase producing organisms.

KEY WORDS

Gram-Negative Bacilli, Extended Spectrum Beta-Lactamase, Amp-C Beta-Lactamase, Amp-C Disk Test, Third Generation Cephalosporins.

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BACKGROUND

Resistant bacteria are emerging worldwide as a threat to favourable outcome in the treatment of common infections in community and settings. Among the wide array of antibiotics, β -lactams are the most widely used agents.¹ The predominant mechanism for resistance to beta-lactam antibiotics in Gram-negative bacteria is by the synthesis of beta-lactamase. Among the beta-lactamase, the production of Extended Spectrum Beta-Lactamase (ESBL) and Amp-C beta-lactamase are the most common.²

ESBLs are enzymes manifesting considerable hydrolytic activity on a wide variety of beta-lactam antibiotics including oxyimino-cephalosporins and aztreonam, and are inhibited

by clavulanic acid.³ Many laboratories have adopted Clinical Laboratory Standard Institute (CLSI) recommendation and only attempted to detect ESBLs in *Escherichia coli*, *Klebsiella pneumoniae*, *K. oxytoca* and *Proteus mirabilis*. Since ESBL genes are transmissible, it is important that ESBLs be tested for in other organisms in hospital and long-term care facility patient populations where ESBLs are encountered.⁴

Amp-C class beta-lactamases are cephalosporinases that are poorly inhibited by clavulanic acid. They can be differentiated from other ESBLs by their ability to hydrolyze cephamycins as well as other extended spectrum cephalosporins.⁵ Genes for Amp-C β -lactamases are commonly found on the chromosomes of the several members of the family Enterobacteriaceae including *Enterobacter*, *Shigella*, *Providencia*, *Citrobacter freundii*, *Morganella morganii*, *Serratia marcescens* and *Escherichia coli*. Plasmid mediated Amp-C β -lactamases has arisen through the transfer of chromosomal genes for the inducible Amp-C β -lactamases onto plasmids. The transfer has resulted in plasmid mediated Amp-C β -lactamases in isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella* species, *Citrobacter freundii*, *Enterobacter aerogenes* and *Proteus mirabilis*.⁶ Unlike chromosome-mediated Amp-C, plasmid-

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Corresponding Author:

Malini Jagannatha Rao,

Specialist cum Assistant Professor,

Department of Microbiology, ESIC-MC-PGIMSR,

Rajajinagar, Bangalore, Karnataka, India.

E-mail: malinirao74@gmail.com

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encoded Amp-C enzymes are almost always expressed constitutively.² Plasmid-mediated Amp-C are associated with potentially fatal errors of false susceptibility on routine susceptibility tests.⁷

Amp-C producing organisms act as hidden reservoir for ESBLs. Their co-existence further complicates the picture, because high level expression of Amp-C β -lactams may mask the recognition of ESBLs. Although reported with increasing frequency, the true rate of occurrence of Amp-C β -lactamases in different organisms including members of Enterobacteriaceae remains unknown. The current Clinical Laboratory Standards Information (CLSI) guidelines do not describe any method for detection of isolates producing Amp-C β -lactamases.

The present study was designed to investigate the presence of ESBLs and Amp-C beta-lactamase enzymes and their co-production in clinical isolates of gram-negative bacilli from a tertiary care hospital.

MATERIALS AND METHODS

This descriptive study was carried out at Employees State Insurance Corporation Medical College and Postgraduate Institute of Medical Sciences and Research (ESICMC-PGIMSR), a tertiary care hospital between April 2011 and June 2011. A total of 205 consecutive non-repeat culture clinical isolates were obtained from various clinical specimens (Table 1), such as urine 88 (42.9%), pus/ wound swab 57 (27.8%), sputum 30 (14.6%), blood 21 (10.2%), stools 06 (2.9%) and miscellaneous samples 03 (1.4%) from both inpatient and outpatients. All the isolates were identified as per recommended procedure.⁸

Antimicrobial susceptibility testing was carried out with Kirby-Bauer's disc diffusion method.⁹ Antibiotic discs used were amoxycillin/ clavulanic acid (20/10 μ g), piperacillin-tazobactam (100/10 μ g), gentamicin (10 μ g), amikacin (30 μ g), ciprofloxacin (5 μ g), trimethoprim-sulfamethoxazole (1.25/23.75 μ g), ceftazidime (30 μ g), ceftriaxone (30 μ g), cefotaxime (30 μ g), cefoxitin (30 μ g), imipenem (10 μ g) and cefuroxime (30 μ g). The zone diameters were interpreted as per the CLSI recommendations.¹⁰ *Escherichia coli* ATCC 25922 strains was used for quality control.

Screening Test for ESBLs

According to the CLSI guidelines, isolates showing inhibition zone size of ≤ 22 mm with ceftazidime (30 μ g), ≤ 25 mm with ceftriaxone (30 μ g) and ≤ 27 mm with cefotaxime (30 μ g) were identified as potential ESBL producers and shortlisted for confirmation of ESBL production.¹¹

Confirmatory Tests for ESBLs

Phenotypic confirmatory test with combination disk (Figure-1). This test requires the use of a third-generation cephalosporin antibiotic disk alone and in combination with clavulanic acid. In this study, a disk of Ceftazidime (30 μ g) alone and a disk of Ceftazidime + Clavulanic acid (30 μ g/10 μ g) were used. Both the disks were placed at least 25 mm apart, center-to-center, on a lawn culture of the test isolate on Mueller-Hinton Agar (MHA) plate and incubated overnight at 37°C. Difference in zone diameters with and without clavulanic acid was measured.

Interpretation: When there is an increase of ≥ 5 mm in inhibition zone diameter around combination disk of

Ceftazidime + Clavulanic acid versus the inhibition zone diameter around Ceftazidime disk alone, it confirms ESBL production.

Screening Test for Amp-C

Isolates showing reduced susceptibility to either of these drugs (ceftazidime or cefotaxime) and cefoxitin.

Detection of Plasmid-Mediated Amp-C Production

Amp-C Disk Test

The test is based on use of Tris-EDTA to permeabilize a bacterial cell and release β -lactamases into the external environment. Amp-C disks (i.e. filter paper disks containing Tris-EDTA) were prepared in-house by applying 20 μ L of a 1:1 mixture of saline and 100x Tris-EDTA to sterile filter paper disks, allowing the disks to dry and storing them at 2 to 8°C. The surface of a Mueller-Hinton agar plate was inoculated with a lawn of cefoxitin susceptible *E. coli* ATCC 25922 according to the standard disk diffusion method. Immediately, prior to use Amp-C disks were rehydrated with 20 μ L of saline and several colonies of each test organism were applied to a disk. A 30 μ g cefoxitin disk was placed on the inoculated surface of the Mueller-Hinton agar. The inoculated Amp-C disk was then placed almost touching the antibiotic disk with the inoculated disk face in contact with the agar surface. The plate was then inverted and incubated overnight at 35°C in ambient air. After incubation, plates were examined for either an indentation or a flattening of the zone of inhibition indicating enzymatic inactivation of cefoxitin (positive result) or the absence of a distortion indicating no significant inactivation of cefoxitin (negative result) (Figure-2).

RESULTS

A total of 205 consecutive non-repeat culture clinical isolates of *Escherichia coli* (n= 87), *Acinetobacter* spp. (n= 29), *Pseudomonas aeruginosa* (n= 27), *Klebsiella pneumoniae* (n=26), *Salmonella typhi* (n= 13), *Enterobacter* spp. (n= 6), *Proteus mirabilis* (n= 5), *Vibrio cholerae* (n= 5), *Providencia* spp. (n= 3), *Citrobacter* spp. (n= 2) and *Shigella* spp. (n= 2) were included in the study. The number of potential ESBL and Amp-C β -lactamase producers detected by the screening test were 135 and 71 isolates respectively.

ESBL Production

Out of the 205 total isolates tested, 135 (65.9%) isolates were resistant to 3GC. ESBL production was seen in 79 (38.5%) of the total isolates. Of the total isolates 56% of *Pseudomonas aeruginosa* n= 14, 50.6% of *E. coli* n= 44, 46.1% of *Klebsiella* spp. n= 12 and 25.8% of *Acinetobacter* n= 8 were found to be ESBL positive (Table 2).

Amp-C Enzyme Production

Of the 205 isolates, 71 showed resistance to either of these drugs (ceftazidime or cefotaxime) and cefoxitin (Table-3) were considered as presumptive Amp-C producers and further confirmed by Amp-C disk test.

Amp-C Disk Test

By Amp-C disk test, 32 (15.6%) isolates showed plasmid Amp-C positive, Table 2.

ESBL and Amp-C Coexistence

This study demonstrated the co-existing phenotypes of both ESBL and Amp-C in 9 (4.4%) isolates, which was noted in *Escherichia coli* 5 (5.7%) and *Klebsiella pneumoniae* 4 (15.3%).

Figure 1. Extended Spectrum Beta-Lactamase Test

Figure 2. Amp-C Disc Test

Clinical Specimen	No. (%)
Urine	88 (42.9)
Pus/ Wound swab	57 (27.8)
Sputum	30 (14.6)
Blood	21 (10.2)
Stools	06 (2.9)
Miscellaneous/ Fluids	03 (1.4)
Total	205

Table 1. Distribution of Isolates from Clinical Specimens

Organism	Total No. of Isolates	ESBL Screen Test Positive No.	ESBL Confirmatory Test Positive No. (%)
<i>Escherichia coli</i>	87	70	44 (50.6)
<i>Acinetobacter</i> spp.	31	24	08 (25.8)
<i>Klebsiella pneumoniae</i>	26	13	12 (46.1)

<i>Pseudomonas</i> spp.	25	21	14 (56)
<i>Salmonella typhi</i>	13	-	-
<i>Enterobacter</i> spp.	6	2	-
<i>Proteus</i> spp.	5	4	01 (20)
<i>Vibrio cholerae</i>	5	-	-
<i>Providencia</i> spp.	3	1	-
<i>Citrobacter</i> spp.	2	-	-
<i>Shigella</i> spp.	2	-	-
Total	205	135	79 (38.5)

Table 2. Prevalence and Distribution of ESBL producing Gram-Negative Clinical Isolates

Organism (n)	Amp-C Screen Test Positive	Amp-C Disc Test Positive No. (%)
<i>Escherichia coli</i> (87)	19	10 (11.4)
<i>Acinetobacter</i> spp. (31)	19	14 (45.1)
<i>Klebsiella</i> spp. (26)	06	05 (19.2)
<i>Pseudomonas</i> spp. (25)	21	02 (8)
<i>Enterobacter</i> spp. (6)	02	01 (16.6)
<i>Proteus mirabilis</i> (5)	03	-
<i>Providencia</i> spp. (3)	01	-
Total (205)	71	32 (15.6)

Table 3. Prevalence and distribution of Amp-C producing Gram-Negative Clinical Isolates

DISCUSSION

Existence of multiplicity of resistance mechanism in gram-negative isolates remains a gray area for most of the clinical microbiologists. Despite the discovery of ESBLs and Amp-C beta-lactamases at least a decade ago, there remains a low level of awareness of their importance and many clinical laboratories have problems in detecting ESBLs and Amp-C beta-lactamases.¹² Organisms over expressing Amp-C β -lactamases are a major clinical concern, because these are usually resistant to all beta-lactam drugs except for cefepime, ceftazidime and carbapenems. Phenotypic variations in the bacterial expression of plasmid encoded Amp-C mediated resistance have to be addressed cautiously. The accurate detection of plasmid mediated Amp-C is important to improve the clinical management of infection and to provide sound epidemiological data.¹³

The isolation of ESBL in the present study is higher 38.5%. This was similar to the ESBL isolation rates noted in different parts of the country (28 - 84%).¹⁴⁻¹⁹ The isolation rate of ESBLs was lower (18.7%) in Vinta Rawat et al. The higher isolation of ESBL in our study might be because of injudicious usage of extended spectrum beta-lactamase in our hospital and can be avoided by adopting appropriate infection control measures. The limitation of this study being the lesser sample size. The present study reveals *Escherichia coli* (50.6%) and *Pseudomonas aeruginosa* (56%) as the major ESBL producers followed by *Klebsiella pneumoniae* (46.1%) and *Acinetobacter* spp. (25.8%). Many laboratories have adopted CLSI guidelines and only attempted to detect ESBLs in *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Proteus mirabilis*.¹¹ Since ESBL genes are transmissible, it is important that ESBLs be tested for other organisms in hospital and long-term care facility patient population where ESBLs are encountered.

Amp-C disk test detected Amp-C beta-lactamase in 32 (15.6%) isolates with highest incidence in *Acinetobacter* spp. (45.1%) followed by *Klebsiella pneumoniae* (19.2%),

Enterobacter spp. (16.6%) and *Escherichia coli* (11.4%). The occurrence of Amp-C beta-lactamase (15.6%) in the present study was lower than that of Manoj Kumar Singh et al (17.7%)²⁰ and Manchanda et al (20.7%).⁵ Vinita Rawat et al (20.8%),¹⁹ Nevine Fam et al (28.3%)¹⁷ and Mohammad Parveen et al (47%),² but was higher than Singhal et al (8%)¹² and Sasirekha Bakthavatchalu et al (5.4%).¹⁸

Carbapenemase producing *Pseudomonas aeruginosa* and *Acinetobacter* spp. may be intrinsically resistant or have acquired resistance to antibiotics due to permeability barrier of the cell surface, multidrug efflux pumps and production of beta-lactamases (Amp-C and ESBL). Because of the production of multiple beta-lactamases by these two organisms and given the fact that Metallo-Beta-Lactamase (MBL) production will hydrolyse all classes of beta-lactams, it can also affect the detection of Amp-C enzyme and ESBL. In our study, Amp-C and ESBL production in *Pseudomonas aeruginosa* was 8% and 56% and *Acinetobacter* spp. showed 45.1% and 25.8%. The comparison between studies becomes difficult, as the patient population in particular centres and the methods of study differ.

The co-existence of ESBL and Amp-C was found in 9 (4.4%), in 5 (5.7%) of *E. coli* and 4 (15.3%) of *Klebsiella pneumoniae*. This co-existence could be due to dissemination of plasmid encoding both Amp-C and ESBL enzymes among Enterobacteriaceae and thus might give false negative tests for the detection of ESBLs. In such situations, it is desirable to develop an ESBL detection test that includes a substrate displaying a higher degree of resistance to Amp-C enzymes like cefepime. Singhal S et al¹² observed the co-existence phenomenon in two (1%) isolates (one each of *Klebsiella* spp. and *E. coli*), Manoj Kumar Singh RK et al²⁰ in 5% and 8% of isolates of Sinha P et al²¹ showed coexistence. The only β -lactam active against co-Amp-C and ESBL producers are carbapenems; however, recently resistance to carbapenems has been increasing which is mostly due to the production of MBL.

Cefoxitin resistance can be used to screen isolates for detecting possible Amp-C β -lactamase production. But lack of permeation of porins has also been reported as one of the resistance mechanisms of cefoxitin in Amp-C non-producers.²² Production of multiple beta-lactamases by clinical isolates has tremendous therapeutic consequences and poses a significant clinical challenge if it remains undetected. In the present study, occurrence of Amp-C and ESBL producers were more commonly seen in clinical specimens from admitted patients, thus shows its nosocomial importance. Although, many phenotypic methods are available for detection of ESBL and Amp-C, gene localisation by genotypic characterisation is considered as gold standard which helps us for epidemiological purpose. Genotypic characterisation could not be carried out in our centre.

CONCLUSION

38.5% and 15.6% of total Gram-negative bacilli in our hospital were found to be ESBL and Amp-C producers respectively. Screening and combined disk tests were effective for ESBL detection. Confirmatory tests that detect cephamycin hydrolysis include the Amp-C disk test and the three-dimensional test. These confirmatory tests based on the detection of cephamycin hydrolysis or Amp-C inhibition will distinguish Amp-C beta-lactamases from ESBLs and porin

mutations. Amp-C disk test is simple, easy to perform and interpretation requires less expertise for the rapid detection. Adoption of this test would make it possible to learn more about the clinical implications of Amp-C β -lactamases and to contain the spread of organisms possessing this resistance mechanism. The limitation of this study was that genotypic studies based on these results could not be carried out in our setup.

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