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Occurrence of ESBL, AmpC and Carbapenemase Producers among Enterobacteriaeceae in Rural Tertiary Care Hospital

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Abstract

Background: Aim of the study was to know the occurrence of ESBL, AmpC and carbapenemases producers among Enterobacteriaeceae by phenotypic disc diffusion tests. *Materials and methods:* A total of 209 isolates belonging to the family Enterobacteriaceae obtained from different clinical samples received in the Department of Microbiology, AIMS. B.G. Ngara were included in the study. ESBL screening was done, followed by phenotypic confirmatory test by CLSI recommended combination disc method. AmpC screening and confirmation was done by phenylboronic acid test and AmpC disc test. Carbapenemase producers were screened and confirmed by CLSI recommended MHT and Remodified Hodge test for KPC detection and double disc synergy test, EDTA disc potentiation test for MBL detection. *Results:* The most common organism isolated was Escherichia coli 101 (48.31%) followed by Klebsiella species 52 (27.27%). Of the 209 isolates of Enterobacteriaceae 24.88%, 1.91% and 7.17% were pure ESBL, AmpC and carbapenemase producers respectively. 5.7% were ESBL and AmpC co-producers, 11.96% were ESBL and carbapenemase co-producers, 2.39% were AmpC and carbapenemase co-producers and 6.22% were combined ESBL, AmpC and carbapenemase co-producers. *Conclusion:* Cefotaxime/clavulanate disc potentiation test detected maximum number of ESBL producers compared to Ceftazidime/ Clavulunate. Cefoxitin–boronic acid detected maximum number of AmpC producers compared to AmpC disc test. Remodified Hodge test is better than MHT in detecting KPC producers. DDST detected more number of MBL producers compared to EDTA disc potentiation test and is a satisfactory and inexpensive method for characterizing the type of carbapenemase producers, when genotypic methods are not available.

Keywords: ESBL; AmpC; Carbapenemases; Phenotypic Methods.

Introduction

Antimicrobial resistance is emerging in the isolates of *Enterobacteriaceae* and is the major threat to the successful treatment of infections in hospitals [1]. The most common mechanism of resistance is the enzymatic inactivation of thebetalactams by a beta-lactamase [2]. Betalactamases are the enzymes produced by microorganisms which can hydrolyze the betalactam ring of beta lactam antibiotics [3].

Organisms producing ESBLs hydrolyze penicillins, cephalosporins and monobactams [3], and are inhibited by clavulanic acid, tazobactam and sulbactam. They are plasmid coded and are easily transmissible from one organism to the other. They are generally derived from TEM and SHV type [4].

AmpC betalactamase confer resistance to a wide variety of beta lactam drugs including beta lactamase inhibitors like clavulanic acid, sulbactam and tazobactam [5]. They are sensitive to cefepime, cefpirome and carbapenems.

It can be plasmid mediated which are typically associated with multi-drug resistance [6] or

chromosomal mediated AmpC, induced in the presence antibiotics such as cefoxitin and imepenem, but poorly induced by 3rd and 4th generation cephalosporins [7].

Both ESBL and AmpC beta lactamases may be produced together by an organism, the effect of plasmid mediated AmpC betalactamases masks the effects of ESBLs which may then be wrongly reported as ESBL negative. This is due to the intrinsic capability of AmpC betalactamases to resist inhibition by clavulanic acid [8]. Boronic acid enhances the detection of ESBL in AmpC producers [8]. Resistance to cefoxitin in *Enterobacteriaceae* indicates AmpC activity [9]. It is important to distinguish between AmpC and ESBL producers, as AmpC producers are resistant to cephamycins and susceptible to fourth generation cephalosporins whereas ESBLs are resistant to fourth generation cephalosporins and susceptible to cephamycin [10].

Carbapenems (ex., imipenem, meropenem, ertapenem and doripenem) are often the antimicrobials of last choice to treat infections due to ESBL and plasmid mediated AmpC producing organisms [11]. KPC confer resistance to all betalactams including penicillins, cephalosporins, monobactams and carbapenems and are inhibited better by tazobactam than clavulanic acid [12].

Class B Metallo beta-lactamases are mostly of VIM- and IMP- types but recently emerged NDM- type is becoming the most threatening carbepenemase [13]. MBL are zinc containing enzymes, they are inhibited by chelating agent such as EDTA and not inhibited by beta lactamase inhibitors [12].

The need for the present study is to compare and to know the advantage of different phenotypic methods in identifying ESBL, AmpC and carbapenemase producing organisms among *Enterobacteriaceae*.

Materials and methods

The present study was conducted in the department of Microbiology, AIMS, B.G. Nagar. Ethical committee clearance was taken from the institution. A total of 209 isolates of *Enterobacteriaceae* from 200 different clinical samples like urine, pus, blood, sputum, high vaginal swab. Organisms were isolated and identified as per standard procedures. All the isolates were further tested for the production of ESBL, AmpC and Carbapenemases.

Methods for detection of ESBL, AmpC, carbapenemases.

Screening test [14]

All isolates were subjected to screening tests to detect ESBL, AmpC and carbapenemase producers.

After adjusting the bacterial suspension to 0.5 MacFarland's units, lawn culture was done on MHA. Amoxicillin-clavulanic acid disc (20µg+10µg) was placed in the center of the petridish and Cefpodoxime (10 µg), Ceftazidime (30µg) disc were placed on either side of Amoxicillin-clavulanic acid disc at a distance of 20mm. Cefoxitin (30µg) disc was placed at a distance of 20mm from Cefpodoxime and Ceftazidime disc. Meropenem (10µg) disc placed at a distance of >25mm from other discs (Fig. 1). Plate was incubated at 35^0C for 16-18hrs.

Interpretation

1. Extension of zone of inhibition of cefpodoxime or ceftazidime towards Amoxyclav disc was taken as ESBL screening positive (Fig. 1).
2. Blunting of zone of inhibition of Ceftazidime towards cefoxitin was taken as AmpC screening positive.
3. Blunting of zone of inhibition of Ceftazidime towards amoxyclav was taken as inducible AmpC positive.
4. Zone of inhibition around Meropenem disc < 21mm was taken as carbapenemase screening positive.

Confirmatory tests

All isolates were subjected to ESBL confirmation test. Those which were screening positive for AmpC and Carbapenemase were also subjected to respective confirmatory tests.

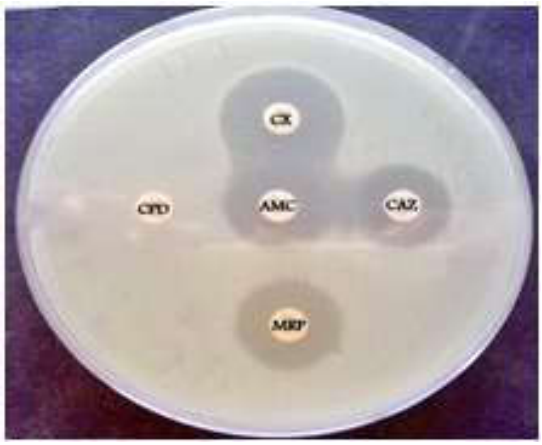

Fig. 1: Screening test for ESBL, AmpC and carbapenemase producers.

1. Confirmatory test for ESBL

Combination disc method [14]

Lawn culture of bacterial suspension was done on MHA. Ceftazidime (30µg), ceftazidime+clavulanic acid(30µg+10µg) and cefotaxime (30µg), cefotaxime+clavulanic acid (30µg+10µg) were placed >30mm apart (Fig. 2). Plates were incubated at 37^{0}C for 16-18 hours.

Interpretation

≥5mm increase in the zone of inhibition of Ceftazidime+clavulanic acid and/or cefotaxime+clavulanic acid discs as compared to Ceftazidime and/or cefotaximediscs alone was taken as ESBL positive. (Fig. 2).

2. Confirmatory tests forAmpC

Combination disc method [14]

Lawn culture of bacterial suspension was done on MHA. Cefoxitin (30 µg) and cefoxitin+ Aminophenylboronic acid disc placed > 30mm apart. The plates were incubated at 37˚C for 16-18 hours.

Interpretation

≥5mm increase in the zone of inhibition of cefoxitin + aminophenylboronic acid when compared to cefoxitin disc alone was taken as AmpC positive.

AmpC disc test:

Lawn culture of *Escherichia coli* ATCC 25922 was done on MHA. Cefoxitin (30µg) disc was placed on it. Test bacterial colony was placed on sterile plain disc of Whatmann filter paper (6mm diameter) adjacent to cefoxitindisc (Fig. 3). The plate was incubated at 37^{0}C for 16-18hours.

Interpretation

Flattening or indentation of the cefoxitin zone of inhibition in the vicinity of test organism disc was taken as positive AmpC disc test. (Fig. 3).

3. Confirmatory tests for Carbapenemase [14]

KPC type carbapenemase [14,15]

Modified and Remodified Hodge test

Lawn culture of *Escherichia coli* ATCC 25922 was done on MHA. Imipenem (10µg) disc and Imipenem (10µg) +zinc (140µg) disc were placed on the inoculated plate. Test strains were streaked like a line with the inoculation loop at right angles to each other from Imipenem and Imipenem+zinc disc, not touching the disc (Fig. 4). The plates were incubated at 37^{0}C for 16-18hours.

Interpretation

Enhancement of growth of indicator strains *E.coli* ATCC 25922 around Imipenem+zinc disc as compared to only imipenem disc was considered as Remodified Hodge test positive (Fig. 4).

Fig. 3: AmpC disc test

Fig. 2: Combination disc method to detect ESBL producer.

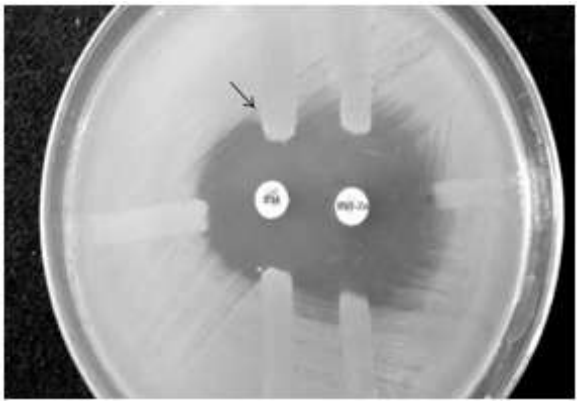

Fig. 3: MHT and Remodified Hodge test.

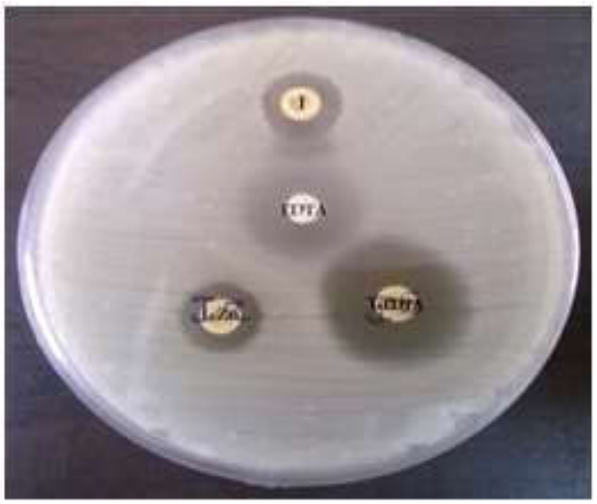

Fig. 5: EDTA disc potentiation and Double disc synergy tests

MBL type carbapenemase [15]

1. Double disc synergy test (DDST)

2. Combination disc method (EDTA disc potentiation test)

Lawn culture was done on MHA. EDTA plain disc (750µg) was placed in the center of the plate. Imipenem (10µg) disc, Imipenem+zinc disc (10 µg+ 140 µg) and Imipenem+EDTA (750 µg) were placed at a distance of 20mm each from EDTA plain disc (Fig. 5). The plates were incubated at 37^0C for 16-18hours.

Interpretation: (Fig. 5)

1. Synergy betweenImipenem disc and plain EDTA disc and Imipenem+zinc disc and plain EDTA disc was taken as double disc synergy test positive.

2. ≥ 3mm decrease in the zone of inhibition of Imipenem+zinc disc as compared to Imipenem disc is taken as combination disc test positive.

3. ≥ 7mm increase in the zone of inhibition around Imipenem+EDTA disc as compared to Imipenem alone was considered as EDTA disc potentiation test positive.

Results

Total of 209 clinical isolates of *Enterobacteriaceae* from different clinical samples like urine, pus, blood, sputum, high vaginal swab collected from out-patients and in-patients admitted in the hospital. Out of 200 clinical samples, majority of the isolates were from urine (55.98%), followed by pus (19.13%), sputum (11.48%), high vaginal swab (6.22%), blood (3.82%), stool (2.87%) and fluid (0.47%).

All isolates were screened for ESBL production, which detected 84 isolates to be ESBL screening positive. Irrespective of screening test results, all the isolates were subjected to phenotypic confirmatory test, which detected 102 isolates to be ESBL positive. 51 (24.4%) were screening positive for AmpC. Combination disc method and AmpC disc tests detected 34 and 31 isolates are AmpC positive respectively.

Of 209 isolates, 115 (55%) were Meropenem resistant and are indicative of carbapenemase production. Out of 115 screening positive isolates, 28 (24.3%) and 30 (26%) were positive for KPC and MBL producers respectively.

Out of 209 isolates, ESBL producers are 102 (48.8%), AmpC 34 (16.3%) and carbapenemase 58 (27.8%) respectively. 52 isolates were ESBL producers, AmpC 4, carbapenemase 15 (8 KPC producers, 7 MBL). 12 isolates were ESBL + AmpC co- producers, 25 ESBL + carbapenemase, 5 AmpC + carbapenemase, 13 ESBL+AmpC+carbapenemase producers. Comparision of various methods in detecting these enzymes are shown in Table.I.

Discussion

Cephalosporins are the first line drugs used in the treatment of infections caused by gram negative organisms. The extensive use of third-generation cephalosporins has resulted in the increased prevalence of ESBL and plasmid- mediated AmpC among these organisms [16].

Carbapenems form an integral part of treatment regimen for serious and multi drug resistant Gram negative bacterial infections. However, there are reports on increasing prevalence of carbapenem resistance in clinical isolates of *Enterobacteriaceae*mainly due to the production of metallo-β-lactamases (MBL) and *Klebsiellapneumoniae* type carbapenemases (KPC) [17].

The present study was conducted to detect the occurrence of β- lactamases among *Enterobacteriaceae* in rural tertiary care hospital. *E.coli* was the most common (48.3%) organism isolated followed by *Klebsiella* species (27.2%), *Citrobacterspp* (7.17%), *Enterobacterspp* (6.2%), *Proteus spp* (4.78%), *Providenciaspp* (4.3%) and *Morganellamorganii* (1.91%) respectively.

Table 1: Comparison of various methods in detection of ESBL, AmpC and carbapenemaseproducers among Enterobacteriaceae.

Methods	Organisms [N=209]						
	E.coli 101(48.3%)	**Klebsiellaspp 52(27.2%)**	**Enterobacterspp 13(6.2%)**	**Citrobacterspp 15(7.17%)**	**Proteus spp 10(4.78%)**	**Providenciaspp 9(4.3%)**	**Morganellaspp 4(1.91%)**
ESBL detection 102(48.8%)							
Screening test 84(40.1%)	48(47.5%)	12(23%)	5(38.4%)	4(26.66%)	7(70%)	6(66.6%)	3(75%)
Combination disc method 102(48.8%)	55(54.4%)	19(36.5%)	8(61.5%)	6(40%)	6(60%)	5(55.5%)	3(75%)
AmpC detection 34(16.3%)							
Screening test 51(24.4%)	21(20.7%)	15(28.8%)	7(53.8%)	4(26.6%)	1(10%)	2(22.2%)	1(25%)
Combination disc method 34(16.3%)	12(11.8%)	9(17.3%)	5(38.4%)	3(20%)	1(10%)	2(22.2%)	1(25%)
Carbapenemase detection 58(27.8%)							
Carbapenemase screening test 115 (55%)	56(55.4%)	24(46.15%)	8(61.5%)	7(46.6%)	9(90%)	7(77.7%)	3(75%)
1) KPC detection 28 (24.3%)							
MHT 20(17.4%)	8(0.79%)	7(13.46%)	4(30.76%)	1(6%)	5(50%)	3(33.3%)	1(25%)
Re- MHT 28(24.3%)	8(0.79%)	6(11.5%)	4(30.76%)	1(6%)	6(60%)	3(33.3%)	1(25%)
2) MBL detection 30 (26%)							
EDTA disc potentiation test 23(20%)	15(14.85%)	5(9.6%)	2(15.38%)	0	1(10%)	3(33.3%)	1(25%)
Double disc synergy test 30 (26%)	12(11.88%)	5(9.6%)	2(15.28%)	2(13.3%)	1(10%)	2(22.2%)	0

In the present study, a total of 209 isolates were screened for the production of ESBL, which showed 84 (40.1%) isolates ESBL screening test positive. Irrespective of the screening test, all the isolates were put for ESBL confirmatory test. Confirmatory test was put using combination disc method, which detected 102 (48.8%) isolates ESBL positive, which is comparable with the study of Dalela G, which detected 135 (61.6%) [18].

In the present study, among the β lactam-inhibitor combination used, CTX and CEC combination detected majority of the ESBL isolates compared to CAZ and CAC. The confirmation of the ESBL production by clavulanic acid inhibition can be difficult in some strains, not only because the activity of the β-lactamase varies with different substrates, but also because the organism may contain additional resistance mechanisms that can mask the presence of the ESBL activity [19]. In the study of Shoorashetty RM et al. [20] and Sturenberg et al. [21] cefepime/clavulanate method could detect maximum ESBL.

Cephamycins are the better screening agents for AmpC production [22]. Confirmatory tests for AmpC was done using combination disc method (Cefoxitin/boronic acid) and AmpC disc test, which detected 34 (35.7%) and 31 (32.6%) respectively. In the study of DL Maraskohle et al., Disc potentiation test using boronic acid detected 59 (35.76%) AmpC producers [23] and study of Vandana KE et al., the PBA (phenyl boronic acid) method detected 24 (58.5%) and 9 (82%) in *E.coli* and *K.pneumoniae* as AmpC positive respectively [24].

In the present study, 20 isolates which were cefoxitin resistant were not AmpC producers. It was observed, though different methods for detection of AmpC β lactamases were used for comparison, that not all cefoxitin resistant isolates were producers of AmpC β lactamases and this range of non producers varied from 20-50% in different studies [7,25]. The resistance in these organisms was considered to be due to the lack of permeation of porins [25]. The limitation of our study was that these isolates needed further evaluation with Modified three dimensional test (M3DT) for AmpC detection. M3DT has an advantage, in this test by using the extract of the organism the effect due to porin mechanism is ruled out and the total effect is due to AmpC β lactamases [23].

Apart from above observation, one Cefoxitin sensitive isolate was a pure AmpC producer. Which is also been reported in the studies of Maraskohle D.L [23] and Hemalatha V [7], where 16.67% and 71.4% of non ESBL Cefoxitin sensitive isolates were AmpC producers. This is because of novel type of AmpC β lactamase with a low level of activity against Cefoxitin which is designated as ACC-1.

In the present study, 115 (55%) isolates were indicative of carbapenemase production. Various studies across the world have reported varying resistance to Imepenem and Meropenem (4-60%) [15].

Out of 115 carbapenemase screening test positive isolates, the MHT detected 20 (17.4%) isolates as carbapenemase producers, while Re-MHT was positive for 28 (24.3%) isolates. Of the above result, Re-MHT detected more KPC than MHT. This is in comparison with the study of AttalRO et al which showed 16 (11.4) isolates to be positive by MHT [34]. Study of SM Amudhan and S Rai detected 113(97.4%), 92 (90.2%) carbapenemase producers respectively [15,27].

Out of remaining 87 screening test positive, which was negative by MHT and Re-modified Hodge test. 23 (20%) were positive by EDTA disc potentiation test and 30 (26%) were positive by double disc synergy test. This can be compared with the study of Srai et al in which EDTA disc diffusion synergy test and combined disc test detected 8 and 9 isolates respectively [15]. Another study by Attal Ro et al showed both methods detected equal numbers of isolates to be carbapenemase positive i.e, 16 (11.4%) among *Pseudomonas aeruginosa* isolates [26].

CLSI recommended combination disc method using clavulanic acid- detected all ESBL but failed to detect ESBL's in the presence of AmpC in 8 isolates. Boronic acid disk potentiation detected all AmpC, and combined enzyme producers correctly compared to AmpC disc test.

In the present study out of 209 isolates, 15(7%) were only carbapenemase (8 KPC and 7 MBL), 25 (11.9%) were combined ESBL and carbapenemase, 5 (2.3%) were combined AmpC and carbapenemase and 13 (6.2%) were ESBL, AmpC, carbapenemase co-producers.

In the study of Oberoi et al coexistence of ESBL and MBL, AmpC and MBL, AmpC & ESBL was seen in 8.79%, 3.67% and 6.59% respectively [28]. A study of Arora et al reported AmpC and MBL coproduction in 46.6% isolates and ESBL and AmpC co production in 3.3% isolates [5].

The present study shows the occurrence of ESBL, AmpC and carbapenemase in 48.8%, 16.26% and 27.7% respectively among *Enterobacteriaceae*. Early detection will help in the management, thus preventing the development and dissemination of drug resistant organisms.

Conclusion

Increased rates of antimicrobial resistance among members of family *Enterobacteriaeceae*, which are known to cause clinically significant infections, suggest monitoring mechanisms of antimicrobial. Phenotypic methods for detection of these resistant mechanisms are faster, cost effective, easier to perform and less labour intensive though it's not confirmatory.

Combination disc method using cefotaxime/ clavulanic acid combination, detected maximum number of ESBL, but it should be performed with other test using boronic acid for the detection of ESBL in the presence of co-production of AmpC.

Cefoxitin-boronic acid method is simple, highly sensitive and easier in detecting AmpC compared to AmpC disc test. Re-MHT using zinc sulphate, detected higher numbers of KPC compared to MHT. Double disc synergy test using zinc sulphate detected maximum number of MBL compared to combination disc method using EDTA.

In order to prevent the spread of these multidrug resistant organisms, it is necessary to identify and detect them routinely in the laboratories using simple phenotypic methods as it helps the clinician to provide appropriate antimicrobial therapy. It is advisable for all the health care settings to have hospital infection control committee with hospital antibiotic policy, with regular updates.

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