ORIGINAL RESEARCH

Phenotypic Detection of Beta-Lactamases in the Enterobacteriaceae Isolates in a Tertiary Care Centre

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ABSTRACT

Background: Multidrug resistance is spreading rapidly among bacterial species around the world. The production of beta-lactamases is responsible for the development of antibiotic resistance. Detecting these resistance mechanisms presents a significant challenge to clinical laboratory. Carbapenemase is a metallo -β-lactamase enzyme that can develop drug resistance. The current investigation sought to detect the synthesis of ESBL (Extended Spectrum Beta Lactamases), Amp C and carbapenemase in Enterobacteriaceae isolated from a tertiary care centre. Objectives: The study aims to analyse the antibiotic susceptibility of Enterobacteriaceae and compare phenotypic approaches to a 12-disk procedure for detecting co-expression of β-lactamases. **Methods:** A cross sectional study conducted with 200 isolates of Enterobacteriaceae members. ESBL, Amp C and MBL production was detected by Phenotypic methods along with 12 disk method. **Result:** Out of 200 samples, 122 (61%) were from adults and male to female ratio was 1.17:1. Most common species isolated was Klebsiella pneumoniae (46.7%). Most of the isolates were resistant to routine antibiotics used. Of the total, 72% were ESBL & 27.5% were AmpC producers. MHT and mCIM identified 43% and 60.5% of carbapenemase producers respectively. 12 disk method identified 21%, 9.5% and 24% of ESBL, AmpC & carbapenem producers respectively. 3.5% of ESBL+AmpC producers, 26% of ESBL & carbapenemase producers, 4.5% of AmpC and carbapenemase producers. 6% isolates were ESBL+AmpC+ Carbapenemase producers. **Conclusion:** Production of beta-lactamases mediates antibiotic resistance in clinical isolates. The development of simple and inexpensive screening methods to detect beta-lactamase production in laboratories is crucial for optimal treatment of critically ill and hospitalized patients, and to control the spread of resistance.

Key words: Amp C, Enterobacteriaceae, ESBL, MBL, resistance.

INTRODUCTION

Multidrug resistance is rapidly increasing worldwide among bacterial species that cause disease in both the community and clinical settings. It is one of the most important public health concerns of the twenty-first century, accompanied by increased morbidity and mortality.[1,2.] Penicillin's discovery signalled the start of the antibiotic era[3]. Beta-lactams are the most often used antibiotics.[4] Resistance to beta lactam antibiotics occurred before beta-lactam penicillin was created.[5]

Acquisition of resistance to these antibiotic agents is mediated through the production of various enzymes called beta-lactamases such as extended-spectrum bata- lactamases (ESBLs), Amp C beta-lactamases and carbapenemases[2].ESBLs are the enzymes most commonly produced by *Escherichia coli* and *Klebsiella pneumoniae* and other *Enterobacteriaceae* species[6]. They hydrolyze oxyimino cephalosporins resulting in the development of resistance to third generation cephalosporins (cefotaxime, ceftazidime and ceftriaxone) and Monobactams (aztreonam).Being plasmid mediated, dissemination of resistance occurs easily among the member of *Enterobacteriaceae*[7]. The current study was conducted to determine different β-lactamases encountered in various Enterobacteriaceae isolated from a tertiary care center.

MATERIALS AND METHODS

Sample collection and processing:

200 clinical isolates of *Enterobacteriaceae* isolated from various non-repetitive clinical samples are used for the study. The samples were processed according to

the standard procedures and organism were identified according to the Grams stain reaction, colony morphology and biochemical reactions.[8]Susceptibility to various classes of antibiotics was done on Mueller-Hinton agar (MHA) by Kirby-Bauer disc diffusion method in accordance with CLSI guidelines.[9]

Phenotypic Methods:

Conventional Methods:

Detection of ESBLs (E-test)

The E-test strips carry two gradients, ceftazidime (TZ) (0.5-32g/ml) on the one end and ceftazidime plus clavulanic acid (TZL) (0.044g/ml) in a different concentration gradient on the other end, along with a fixed concentration of clavulanic acid (4g/ml). A broth culture of the test isolate was lawn cultured over the Mueller Hinton agar and allowed to dry. The isolate is considered ESBL producer if there is appearance of a phantom zone or deformation of the TZ eclipse or when ceftazidime MIC is reduced by >3 log2 dilution (ratio TZ/TZL,>8) in the presence of clavulanic acid as per the manufacturer's guidelines.[10] Beta lactamase negative *Escherichia coli* ATCC 25922 was used as the negative control and ESBL producing *K.pneumonia* ATCC 700603 was used as positive controlthroughout the study.

Detection of Amp C: (Disc approximation test.)

0.5 McFarland bacterial suspension was prepared from an overnight blood agar plate and the surface of the MHA plate is inoculated using this suspension as per the standard disc diffusion method. A 30µg ceftazidime disk is placed at the center of the MHA plate. Then, 10µg imipenem, 30µg cefoxitin, and 20/10µg amoxicillin-clavulanate discs were placed at a distance of 20mm from the ceftazidime disk. The plate is incubated for 24 hours. The plate is examined for any blunting or flattening of the zone of inhibition between the ceftazidime disk and the imipenem, cefoxitin and amoxicillin-clavulanate disk. The result is considered positive if there is any blunting or flattening of the zone.[11]

Test for

For carbapenemase:

Modified Hodge Test (MHT)

Detection of carbapenemase production was performed using the Modified Hodge Test on Mueller-Hinton agar. 0.5 McFarland of negative control *E. coli* ATCC 25922 as uniformly swabbed onto Mueller Hinton Agar (MHA) and test isolate was streaked as a straight line from edge of the Imipenem disc (10µg), to the edge of the plate. After the incubation of overnight, indentation in the growth of the negative control towards the imipenem disc on either side of the test isolate as considered as positive for theproduction of metallo-beta lactamase by the test. [12]

Modified Carbapenem Inactivation Method (m CIM):

As per CLSI guidelines, the mCIM test is recommended for carbapenemase detection replacing MHT.

Using a sterile inoculating loop, 10µl of test organism was added into a tube containing 2 ml of tryptic soy broth (TSB), the bacterial suspension was vortexed for 10 to 15 seconds. Next, a 10mg meropenem disk was aseptically added into the bacterialsuspension. The tube was then incubated for 4 hours at 35°C in ambient air. Just prior to completion of the 4-hour carbapenem inactivation step, a suspension of the mCIM indicator organism (*Escherichia coli* ATCC 25922) with turbidity equivalent to a 0.5 McFarland standard was prepared, and the surface of MHA plate was inoculated using the procedure for standard disk diffusion susceptibility testing.[9] The meropenem disk was then removedfrom the TSB bacterial suspension using a 10µl inoculating loop; the loop was dragged along the edge of the tube during removal to remove excess liquid, and the disk was placed on the inoculated MHA plate, which was then incubated in an inverted position for 18 to 24 h at 35°C in ambient air. A zone diameter of 6-15mm or the presence of pinpoint colonies within a zone is considered carbapenemase positive. The zone diameter of more than or equal to 19 mm clear zone is considered carbapenemase negative.

12-Disk method:

Detection of different beta-lactamases such as ESBL, AmpC and Carbapenemase simultaneously using a combination of antibiotic discs in a single 150mm Mueller Hinton agar plate. The broth culture of the test isolate was lawn cultured on a 150mm Muller Hinton Agar plate incubated it at 37ºC for 18 hours. Results were interpreted as per CLSI guidelines.

Isolates showing an increase in zone size of more than / equal to 5 mm with clavulanate compared with plane ceftazidime/cefotaxime disks are interpreted as ESBL positive. Isolates showing sensitivity to cefepime disk and resistance to cefoxitin disks are interpreted as Amp C positive. Isolates showing resistance to the carbapenem agents (imipenem, meropenem, ertapenem) are interpreted as Carbapenemase positive.[2]

RESULT

List of organisms isolated from clinical samples and antimicrobial resistance pattern:

In our study, most common species of *Enterobacteriaceae* isolated was *Klebsiella pneumoniae* i.e. 93 (46.7%), followed by *E. coli* 73 (36.5%). Other species isolated were *Klebsiella oxytoca, Proteus spp., Enterobacter cloacae, Citrobacter spp., and Serratia spp.*

Of the total, 183 (91.5%) isolates were resistant to ceftriaxone. More than 65% of the isolates were resistant to all commonly used antibiotics, except for colistin and tigecycline for which 11 (5%) and 46 (23%) of the isolates were resistant.

87 out of 93 (93%) *K. pneumoniae* isolates were resistant to ceftriaxone, followed by cefoxitin i.e. 84 (90%) isolates. All the isolates of *K.oxytoca, P.mirabilis and E.cloacae* are resistant to

ceftazidime. *P.vulgaris* showed 100% resistance to all the antibiotics used, except for ceftazidime, colistin, and tigecycline. All the isolates of *C.koseri* are resistant to all the antibiotics used except for colistin and tigecycline. *S. marcescens* showed 100% resistance to all the antibiotics used including colistin & tigecycline

Phenotypic detection:

1. E-test for ESBLs:

Of the total, 72% of isolates tested positive and 28% tested negative for E-test.

Table 1: Detection of ESBL by E-Test

Response	Number of patients (n=200)	Percentage %
Positive	144	72.00
Negative	56	28.00

2. Disc approximation test (DAT) for AmpC:

Out of 200, 27.5% showed positive and 72.5% showed negative for disc approximation test.

Table 2: Results of Disc approximation test

Response	Number of patients (n=200)	Percentage
Positive	55	27.5
Negative	145	72.5

3. Modified Hodge Test (MHT) for Carbapenemase:

Among the total, 43% were carbapenemase producers and 57% were non-carbapenemase producers.

4. Modified Carbapenem Inactivation Method (mCIM):

Out of 200, 39.5% tested negative for mCIM and 60.5% tested positive for mCIM.

Among the 2 tests used to detect carbapenemase, MHT could detect 86 (43%) of the isolates and mCIM could detect 121 (60.5%). Sensitivity and specificity of MHT test was 71% and 100% respectively.

Beta-lactamases and co-producers detected by individual phenotypic method:

As shown in the table-10, ESBL+ Carbapenemase co-producers were maximum i.e.61 (30.5%) followed by 44 (22%) ESBL producers, 30 (15%) carbapenemase producers. 23 (11.5%) isolates were positive for all 3 beta-lactamases i.e. ESBL + AmpC+ Carbapenemase. 10 (5%) isolates tested negative for beta-lactamases.

Table 3: Distribution of ESBLs, Amp C, Carbapenemase and their co-production among the isolates detected by phenotypic methods

Beta-lactamases	No. isolates (n=200)	Percentage (%)
ESBL	44	22
AmpC	9	4.5
CARBAPENEMASE	30	15
ESBL + AMPC	16	8
ESBL+ CARBAPENEMASE	61	30.5
AMPC+ CARBAPENEMASE	7	3.5
ESBL+AMPC+ CARBAPENEMASE	23	11.5
NIL	10	5
TOTAL	**200**	**100**

Among the isolates, maximum ESBL production was seen in K. oxytoca & P. vulgaris i.e. 2 (40%) followed by E. coli, E. cloacae and K. pneumoniae i.e. 20 (27.3%), 2 (20%) & 16 (17%) respectively. Maximum AmpC production was encountered in E. cloacae i.e. 1 (10%). 3 (60%) isolates of K. oxytoca were MBL producers. C. freundii showed maximum co-production of ESBL+AmpC and ESBL+MBL i.e. 1 (25%) and 3 (75%) respectively. 1 (20%) isolate of P. vulgaris showed co-production of ESBL+AmpC+Carbapenemase.

Table 4: Distribution of ESBL, AmpC and MBL among the isolated organisms.

Organism		ESBL	Amp C	MBL	ESBL + Amp C	ESBL + MBL	Amp C + MBL	ESBL + Amp C + MBL
Klebsiella pneumoniae	N=93	16	04	14	15	29	03	10
	%	17	4.3	15	16	31.1	3.2	10.7
Escherichia coli	N=73	20	04	09	05	20	02	10
	%	27.3	5.4	12.3	6.8	27.3	2.7	13.6
Enterobacter cloacae	N=10	02	01	00	01	04	01	01
	%	20	10	00	10	40	10	10
Proteus mirabilis	N=08	02	00	03	00	01	01	00
	%	25	00	37.5	00	12.5	12.5	00
Klebsiella oxytoca	N=05	02	00	03	00	00	00	00
	%	40	00	60	00	00	00	00
Proteus vulgaris	N=05	02	00	00	01	01	00	01
	%	40	00	00	20	20	00	20
C. freundii	N=04	00	00	00	01	03	00	00
	%	00	00	00	25	75	00	00
C. koseri	N=01	00	00	00	00	00	00	00
	%	00	00	00	00	00	00	00
S. marcescens	N=01	00	00	00	00	00	00	00
	%	00	00	00	00	00	00	00

5. 12 Disk method:

Out of 200 isolates tested by 12 disk method, 11 (5.5%) were negative for all beta- lactamase production. Of the total, 42 (21%) isolates were pure ESBLs, 19 (9.5%) were pure AmpC and 48 (24%) were pure carbapenems producers. In 7 (3.5%) and 52 (26%) isolates, ESBLs coexisted with AmpC and carbapenemase respectively. In 9 (4.5%) isolates, AmpC co-existed with carbapenemase. 12 (6%) isolates were positive for all 3beta-lactamases.

Comparison of individual methods and 12-disk method:

Individual methods could detect 9 (4.5%) & 30 (15%) pure AmpC and pure carbapenemase producers respectively. Whereas 12-disk method could detect 19 (9.5%) & 48 (24%) pure AmpC & Carbapenemase producers respectively. Individual method coulddetect 61 (30.5%) ESBL+carbapenemase co-producers, whereas 12 disk method could detect 52 (26%) ESBL+carbapenemase co-producers.

Table 5: Comparison of 12 disk method with individual phenotypic tests

Beta-lactamases	Individual methods		12 disk method	
	Total (n=200)	%	Total (n=200)	%
ESBL	44	22	42	21
AMPC	9	4.5	19	9.5
CARBAPENEMASE	30	15	48	24
ESBL + AMPC	16	8	7	3.5
ESBL+ CARBAPENEMASE	61	30.5	52	26
AMPC+ CARBAPENEMASE	7	3.5	9	4.5
ESBL+AMPC+ CARBAPENEMASE	23	11.5	12	6
NIL	10	5	11	5.5
TOTAL	**200**	**100**	**200**	**100**

DISCUSSION

The beta-lactamases emerged as the most common cause of antibiotic resistance among gram negative bacteria worldwide. ESBLs represent a major group of beta- lactamases, currently being identified worldwide in large numbers along with inducible Amp C beta-lactamases. MBL genes have spread from *P.aeruginosa* to members of *Enterobacteriaceae*. Reports indicate that the carbapenemase producing *Enterobacteriaceae* seem to be increasing in number in recent years. These enzymes are plasmid mediated. Multidrug resistance and even pandrug resistance are characteristic feature of strains producing these enzymes. This leaves us with a very narrow spectrum ofdrugs for treatment.

The risk factors leading to the infection with multidrug resistant organisms include arterial or urinary catheterization, increased length of hospital stay, prolonged stay in the intensive care unit and inappropriate use of various broad-spectrum antibiotics.

The prevalence of ESBL, Amp C and MBL producers are found to vary greatly in different geographical areas and in different institutes. In the current study, an attempt was made to know the prevalence of ESBL, Amp C, MBL and their co-production in Enterobacteriaceae isolates by various phenotypic methods and their antibacterial susceptibility pattern.

Out of 200 subjects included in the study, maximum number of beta-lactamases were isolated from adults (19-59 years) i.e. 122 (61%). These findings were comparable with study conducted by Ayesha Ansari et.al.[13], where maximum cases were of third and fourth decade.

In our study, out of 200 isolates of *Enterobacteriaceae*, 108 (54%) were isolated from males and 92 (46%) from females with male to female ratio 1.17:1. The findings were comparable with the study by Sood S et.al. where males were more than females [14] . But is in contrary with the findings of Baby Padmini et al [15]who found female predominance in their study.

In our study, most of the beta-lactamase producing *Enterobacteriaceae* spp. were isolated from Pus sample (41%), followed by urine (21.5%), sputum (15%) and blood (8%). This finding differs from that of the other studies, Kumar D et al.[16] and Sood S [17] where most of the organisms were isolated from urine (41.6%) and blood (25%) respectively.

Distribution of beta-lactamase producing Enterobacteriaceae spp.

In the present study, out of 200 isolates of *Enterobacteriaceae*, most common isolate was *Klebsiella spp.* (49%), followed by *E. coli* (36.5%), which is in consistence with the finding of the study Franklin et al [19]. Contrary to our study results, few authors found *E. coli* as most common isolate followed by *Klebsiella spp.*[2,15, 18, 20]

Table 6: Comparison of organisms isolated in various studies

Studies	*Klebsiella spp.*(%)	*E. coli* (%)	*Proteus spp* (%)	*Enterobacter* spp(%)	*Citrobacter* spp.(%)	*Serratia spp* (%)
Ratna et al[18] (2003)	35.4	53.7	-	7.69	-	3.8
Franklin et al[19] (2006)	9.5	7.3	-	8.0	8.8	1.4
Sridhar Rao et al[20] (2008)	13.72	35.7	-	4.4	-	-
Valsan C et al[2] (2013)	33.1	58	0.75	7	1	-
Swaminathan A et al[15](2016)	13	26	-	-	-	-
Present Study	49	36.5	6.5	5	2.5	0.5

In our study, 91.5% isolates were resistant to ceftriaxone. All strains showed multidrug-resistant profile. 5% and 23% of the isolates were resistant to colistin and tigecycline as well. These findings are in consistent with a study by Gonzalez d et. Al [21].In contrary to our study where only 27% isolates were sensitive to imipenem, 100% sensitivity to imipenem was observed in a study by Kumar D et al.[16] 100% sensitivity of higher drugs like colistin and tigecycline was noted in a study by Sood S [17], where as in our study, 95% and 77% sensitivity were observed for colistin and tigecycline respectively. This is the dangerous finding indicating the emergence of organism with resistance to higher, reserved drugs like tigecycline and colistin.

Out of 200 *Enterobacteriaceae* isolates screened 72% were ESBL, 27.5% were Amp C and 60.5% were MBL producers.

Govinda swamy A et al., conducted a study on prevalence of β-lactamases which showed that the overall prevalence of bata-lactamases i.e. ESBL, AmpC and carbapenemase production was found to be 88.3%, 42.2% and 65.1%[21]. In a study by Valsan C et al[2], 60% were ESBL producers, 10% were AmpC producers and 12.6% were carbapenemase producers. Prevalence of ESBLs in our study was found to be higher compared to the previous studies done in Mumbai (53%)[6] Bhopal (48.27%) [23], Hyderabad (19.8%)[7], Pondicherry (66.7%) [24] and Bijapur (32.1%).[22]

Prevalence of AmpC producers among Enterobacteriaceae by disc diffusion method was 36.5% in a study conducted in Uganda [25], 46.3% in Pakistan and 42.2% in India (2019). Our study had lower prevalence rate compared to these studies and other studies done in India [21,26]. These findings are in contrary to a study conducted by Rodrigues et al [6] where only 7% of the isolates were Amp C producers.

Our study had 60% carbapenem-resistant *Enterobacteriaceae* (CRE). In a study conducted in China between 2014-16 isolated 18% of CRE showing increasing trend of prevalence [27]. Similar

study conducted in India between 2014-16 had 9.20% of carbapenem resistant isolates [28]. On comparing with our study, there is a drastic increase (6fold) in Carbapenem resistant isolates between 2016-2019. This is an alarming finding and challenge to current antibiotic era.

In our study, MHT could detect 43% of carbapenemase producing isolates whereas mCIM test detected 60.5% of the total. This could be because of the reason that MHT was found to perform better for other carbapenemase like KPC, SME, IMI compared to NDM [15,29].

Co-production of ESBLs, AmpC and MBLs:

In current study, co-production of ESBLs and AmpC were encountered in only 8% of the isolates. This is contrary to the studies by Singla P et al.[30] & Kumar V et al.[31] whereauthors observed 29% and 24.5% of the isolates were co-producers of ESBL & Amp C.

ESBL and carbapenemase co-production were observed in 30.5% of the isolates outof 200 in our study. Our findings showed higher prevalence of ESBL & carbapenemase co-producers compared to a study by Ibrahim Y et al. where in 14.1% of the isolates co- production was seen[32].

In our study co-production of AmpC with carbapenemase was low (3.5%) which is in consistence with the previous study which showed 7.5%[33]. However, a study in this contrary showed high prevalence i.e. 48.5% of AmpC and carbapenemase co-production[31].

In our study, maximum ESBL production is seen in *K. oxytoca* (40%) & *P. vulgaris*(40%) followed by *E. coli* (27.3%), *E. cloacae* (20%) and *K. pneumoniae* (17%). In contrary, Neelam Taneja et al [32] found that 51.2% ESBL producing isolates were *K.pneumoniae* and 40.2% were *E. coli.*

In present study, maximum AmpC production was encountered *E. cloacae* (10%). In a study conducted by Soon N et al[11], out of 82 isolates, *E. coli* was 69.51% and *Klebsiella pneumoniae* were 30.48%.

Li Y et al. found that among Carbapenem producing isolates, *K. pneumoniae* and *E. coli* were 2 most common spp accounting for 52.9% and 17.5% of total respectively[27] . This differs compared to our study findings where *K. oxytoca* is common isolate among carbapenemase producers followed by *E. coli.*

12-disk method:

In our study, 12-disk method could isolate 21% of pure ESBLs, 9.5% of pure AmpCproducers, 24% of pure carbapenemase producers. It also detected the co-producers. 3.5% of ESBL producers were also positive for AmpC production. In 26% of isolates ESBLs co-existed with carbapenemase. 4.5% of the isolates were positive for both AmpC and carbapenemase. Of the total, 6% of the isolates showed the co-production all 3 beta- lactamases i.e. ESBL, AmpC & carbapenemase.

Similar study was conducted by Valsan C et al[2]. They found 46% of pure ESBL producers which is double the rate of our study and 4.5% of pure AmpC producers which is less compared to our study. They found 3% and 9% of the isolates as pure Carbapenemase producers and co-producers of Carbapenemase and ESBL respectively. This is not in consistent with the current study where rates of pure carbapenemase and its co production with ESBLs are as high as 24% and 26% respectively. They found the higherrate of ESBL and AmpC co-producers (5%) compared to our study (3.5%). They had no AmpC and carbapenemase co-producers and co-existence of all 3 beta-lactamases.

On comparing 12-disk method to phenotypic tests, results were comparable except for the detection rate of pure AmpC and ESBL-AmpC co-producers. 12 disk method couldn't isolate all ESBL+AmpC co-producers. Presence of AmpC masked the effect of beta-lactamase inhibitor enhancement which is used for the detection of ESBLs. As a result, presence of ESBLs couldn't be detected and were identified as only AmpC producers by this method. Similarly, detection rate of pure Carbapenemase and its co-production with ESBL, 12 disk method couldn't detect much of carbapenemase and ESBL co-producers. It failed to detect the ESBL in the presence of carbapenemase and were detected as pure carbapenemase. However, this should not be a major issue for clinical laboratories as the treatment options in both the conditions are similar.

With our findings, individual methods could detect co-producers better, whereas 12 disk method could detect pure beta-lactamases producers better. But in general, 12 disk method is easy and cost-effective, that can be adopted by laboratories for routine testing where there are no facilities for automated and molecular testing.

CONCLUSION

Production of beta-lactamases mediates antibiotic resistance in clinical isolates. The development of simple and inexpensive screening methods to detect beta-lactamase production in laboratories is crucial for optimal treatment of critically ill and hospitalized patients, and to control the spread of resistance.

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