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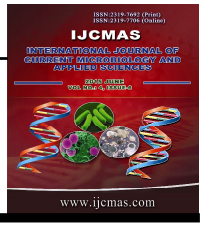

Original Research Article

Phenotypic Detection Methods of Carbapenemase Production in Enterobacteriaceae

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A B S T R A C T

Carbapenems are frequently used as a last resort antibiotic in the treatment of infections due to multidrug-resistant *Enterobacteriaceae*. Their alarming increase worldwide is worrisome and is left with very restricted therapeutic alternatives. This study was pursued to identify phenotypically the presence of carbapenemase producing *Enterobacteriaceae* among various clinical isolates received in the central microbiology laboratory in a tertiary care 1600 bedded hospital. Third generation resistant cephalosporin isolates were further studied for carbapenems resistance by disk diffusion method. Antibiotic susceptibility testing to various classes of antibiotics was analysed. Modified Hodge test (MHT) and combined imipenem –EDTA disk diffusion test were employed for carbapenemase detection.A total of 114 of 200 third generation resistant cephalosporin isolates were found to be carbapenem resistant to either of imipenem, meropenem or ertapenem disks. Cross resistance to other classes of antibiotics like fluoro-quinolones (67%), aminoglycosides (80%) and β-lactam-β–lactamase inhibitor combination drugs (61%) was observed. Screening for carbapenemase producers by MHT yielded 30.5% and for Metallo-β-lactamases by inhibition with EDTA 32.5% and 43% were negative by both methods. A total of 62% were carbapenemase producers identified by both the screening methods. Use of both MHT and combined disk test with EDTA as a screening method can increase the sensitivity of detection of carbapenemases and can aide timely intervention to initiate infection control practices and thereby improve patient outcome.

Introduction

Carbapenems are frequently used in the treatment of infections due to multidrug-resistant *Enterobacteriaceae*. The alarming increase in the rate of carbapenemase producing *Enterobacteriaceae* clinical isolates over the last few years have led to pan drug resistant bacteria with limited therapeutic options. Carbapenem resistance in *Enterobacteriaceae* is attributed to class A carbapenemases (*Klebsiella pneumonia*e

carbapenemase KPC types), the class B or metallo β-lactamases (MBLs) (VIM, IPM, and NDM types), and the class D oxacillinases (e.g., OXA-48-like enzymes). (Queenan *et al.,* 2007)

The laboratory identification of carbapenemases needs to be modified as an increasing assortment of β-lactamases is reported in members of the Family *Enterobacteriaceae*. Both MBL and KPC carbapenemases are known to co-exist and are increasingly recovered from clinical specimens leading to difficulty in differentiating and identifying these enzymes by phenotypic testing. These enzymes hydrolyze almost all β-lactam antibiotics, and hence the detection of each one of them can be masked by the expression of the other.

MBL and KPC Carbapenemases are mostly encoded by mobile transposon and/or integron determinants resulting in faster dissemination to the other members of *Enterobacteriaceae* with limited therapeutic choices (Athanassios Tsakris *et al.,* 2010).

Therefore it is essential to detect and differentiate class A and B carbapenemases among *Enterobacteriaceae* isolates in the clinical laboratory that may provide substantial information before application of the more expensive molecular techniques. This study focused on the simple phenotypic detection methods for carbapenem resistance for aptmediationin reducing the spread of such isolates and further management.

Materials and Methods

This prospective cross sectional study was initiated after obtaining approval from the Institutional Ethical Committee (CSP/12/JAN/08). A total of 200 third generation cephalosporin resistant isolates that showed resistance to either of the carbapenems namely imipenem, ertapenem or meropenem by disk diffusion method were selected for the study. The identity of the isolates was confirmed using a set of in house biochemical tests and Vitek 2 GN ID (BioMerieux, France) with appropriate quality control.

Antimicrobial susceptibility:

Antimicrobial susceptibility was tested according to CLSI recommendations. The diffusion method on Mueller-Hinton agar (Himedia, Mumbai) was used to test susceptibility to Ampicillin (A-10µg), Cephalexin (Cn-30µg), Cefuroxime (Cxm-30µg), Ceftazidime (Caz-30µg), Cefotaxime (Ce -30µg), Cefepime (Cpm -30µg), Imipenem (I-30 µg), Meropenem-(Mem-10µg), Ertapenem-(Etp-10µg), Ciprofloxacin (Cip-5 µg), Ofloxacin (Of-5µg), Amikacin (Ak-30µg), Netilmicin (Net-30µg), Tigecycline-(Tgc-15µg), Polymyxin (Pb 300U), Piperacillin/ Tazobactum- Pit (Piperacillin-100µg, Tazobactum-10µg) and Cefaperazone/ Sulbactam- Cfs (Cefaperazone-75µg, Sulbactam- 30µg).

Screening for ESBL production

Those strains that were resistant to cefotaxime and or ceftazidime with reduced susceptibility were included as ESBL producers.

Screening for Amp C β-lactamase production

Screening for Amp C β-Lactamase production was done by using Cefoxitin (30µg) and were considered resistant when inhibition zone was ≤ 16 mm size according to CLSI guidelines.

Phenotypic detection of carbapenamase production by Modified Hodge test (MHT)

All the isolates were subjected to Modified Hodge test as per CLSI guidelines**.** A lawn culture of the 1:10 dilution of *Escherichia coli* ATCC 25922 as recommended by CLSI was done on Mueller Hinton agar plate and a 10-µg meropenem susceptibility disk was placed in the centre of the test area. The test organism was then streaked in a straight line from the edge of the disk to the edge of the plate. Four strains were tested on the same plate with one disk and were incubated overnight at 35°C ± 2°C in ambient air for 16–24 hours.

Interpretation was done after 16–24 hours of incubation. Positive Modified Hodge test showed a clover leaf-like indentation of the *Escherichia coli* 25922 strain growing along the test organism growth streak within the disk diffusion zone indicating production of carbapenemase and a negative test showed no growth of the *Escherichia coli* ATCC 25922 along the test organism growth streak within the disk diffusion.

Combined Imipenem- EDTA disk test for detection of Metallo-β-lactamases (MBL)

All isolates were tested with the combined imipenem-EDTA test as per Yong *et al.*2009with appropriate positive and negative controls**.** Test organisms were inoculated on to Mueller Hinton agar plates as per CLSI guidelines. A 10-µg imipenem disk and imipenem (10-µg) - EDTA (750µg) combined disk were placed on the plate (Hi-Media, Mumbai) and incubated for 16-18 hours at 35°C. The increase in inhibition zone with the imipenem and EDTA disk ≥7 mm than the imipenemdisk alone was considered as a Metallo β-Lactamase (MBL) positive strain.

Results and Discussion

Study design: This study was conducted in the Department of Clinical Microbiology laboratory in Sri Ramachandra medical centre which caters to 1600 beds. 200 isolates of Family *Enterobacteriaceae* resistant to third generation cephalosporins were included and resistance to either of the carbapenems namely imipenem, ertapenem or meropenem by disk diffusion method were selected for the study.

Distribution of *Enterobacteriaceae*: The Following isolates were tested: *Escherichia coli* (n=123), *Klebsiella pneumoniae* (n=43), *Enterobacter cloacae* (n= 12), *Proteus vulgaris* (n=8), *Providencia rettgeri* and *Citrobacter freundii* (n=5) each and *Proteus mirabilis* (n=4).

Source specimen of the isolates: The source distribution of specimens included 153 strains from urine, 34 from exudates (8 wound swabs, 3ear swabs, 2 peritoneal fluid, 1 tissue sample, & 20 pus samples) 10 from blood and 3 from respiratory secretions (2 Non-Bronchoalveolar lavage and 1 endotracheal secretion).12 strains were collected from intensive care units and 188 from various wards.

Antibiotic susceptibility pattern

Resistance to fourth generation cephalosporins - cefepime was 77% (155/200), β-lactam β-lactamase inhibitor combination drugs like cefaperazone-sulbatum and piperacillin – tazobactum showed a resistance rate of 44% (89/200) and 60% (121/200) respectively. Cross-resistance to other classes of antimicrobial agents was observed in this study. Among aminoglycosides, amikacin exhibited 58% (116/200), netilmicin 68% (137/200) and tobramycin 75% (151/200) whereas

quinolones, showed 75% (150/200) to ciprofloxacin and 61% (133/200) to ofloxacin. Minimum resistance was noted with Polymyxin- B 6% (12/200) followed by tigecycline 10.5 % (21/200).

Detection of Carbapenemase production

About 62% (114 of 200) carbapenem resistant isolates tested positive for production of carbapenemases by both MHT and MBL screen. Among the 114 strains, 30.5 % (N=61) were detected by MHT and 32.5% (N=65) by MBL screen. Both MBL and MHT screen were positive in 12 (6%) isolates (Table 1). Authors from different parts of India have reported varying resistance rates of carbapenem in *Enterobacteriaceae* ranging from 5.75% to 51% in various gram negative bacilli over a decade. (Gupta *et al.,* 2006; Wattal *et al.,* 2008; Deshmukh *et al.,* 2011; Priya Datta *et al.,* 2012; Shanthi *et al.,* 2013; Shanmugam *et al.,* 2013; Rai *et al.,* 2014).

Carbapenemase producers are increasingly reported across the globe in Family *Enterobacteriaceae.* Mostly, three types of carbapenemases are now commonly identified namely Ambler class A of the KPC type, class B of the NDM-1, IMP, and VIM types, and class D of the OXA-48 type (Yan *et al.,* 2002; Lee *et al.,* 2001)**.** Many techniques are being used for detecting production of carbapenemases, from phenotypic to advanced molecular-based ones.

The main advantage of MHT is that different carbapenemase classes can be recognised in a single plate and the disadvantage is it cannot discriminate between various classes of carbapenemases and leads to false positives for Amp C and ESBL isolates (Lee *et al.,* 2001).

Routine disk susceptibility testing indicates only resistance to carbapenems and cannot always detect Metallo-β-lactamases (MBL). This resistance can be mediated by multiple mechanisms. Detection of MBL is imperative as it calls for preventive measures to halt the spread of multi drug resistant (MDR) strains. The MIC to carbapenems may not be considerably raised in MBL-producing strains and it is important to implement simple screening procedures.

Carbapenemase production was witnessed at a higher rate in *Enterobacter cloacae* and *Providencia rettgeri* (83%) when compared to 63% in *Proteus vulgaris,* 60% in *Citrobacter freundii* and *Klebsiella pneumoniae*, 52% in *Escherichia coli* and 50% in *Proteus mirabilis.* This was marginally higher than the rates observed by (Wattal *et al.,* 2010) in a surveillance study in 2008 in ICU isolates and noted that carbapenemase production in *Escherichia coli*, Klebsiella spp., as 31&51% respectively.

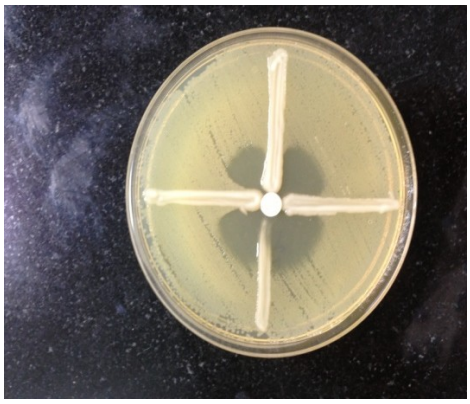
86(43%) isolates tested negative by both methods in spite of being resistant to carbapenems by disk diffusion. The likely explanation could be over production of ESBL, or Amp C hyper producers with porin loss. Organisms that produce carbapenem-hydrolyzing enzymes frequently produce more than a single β-lactamase (Beth Rasmussen *et al.,* 1997). Amp C β -lactamase production was screened by cefoxitin disk diffusion and found to be 61%. 39 isolates (19.5%) were negative for all the screening tests namely for carbapenemase and Amp C producers.

Class A and class D carbapenemase producers were easily detected by the MHT but not so with NDM-MBL producers.

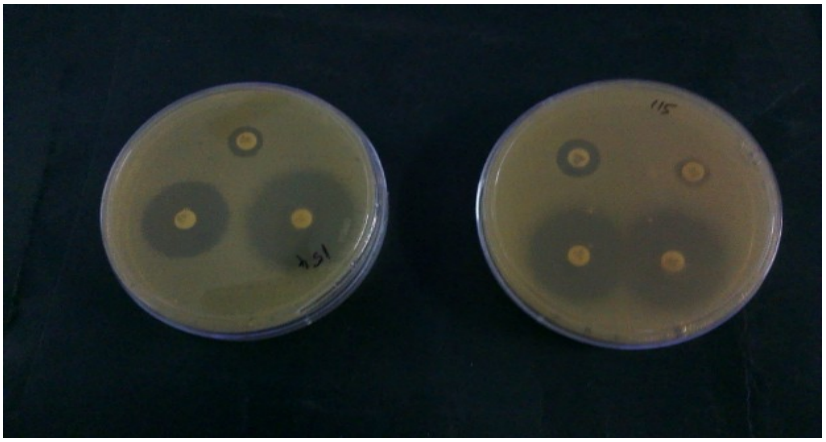
Table.1 Distribution of various types of β- lactamases in Family Enterobacteriaceae

Organisms	MHT Positive	MBL Positive	Both MHT & MBL positive	Amp C Positive	Carbapenemase + Amp C+ ESBL β- lactamases
Escherichia coli (N=123)	27	43	4	40	6
Klebsiella pneumoniae (N=43)	17	11	6	15	1
Enterobacter cloacae (N=12)	8	3	3	6	1
Citrobacter freundii (N=5)	3	1	2	2	0
Providencia rettgeri (N=5)	2	3	0	3	0
Proteus mirabilis (N=4)	1	1	0	1	0
Proteus vulgaris (N=8)	3	3	0	3	1
Total (200)	61	65	12	70	9

Fig.1 Detection of carbapenamase by MHT and Combined imipenem- EDTA disk test



1a) Modified Hodge test-Positive clover leaf pattern



1b) Combined disk test with increase in zone size for imipenem-EDTA disk than imipenem disk alone

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