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Phenotypic differentiation of class A and class B Carbapenemases in Enterobacteriaceae

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ABSTRACT

Appropriate detection of Carbapenem Resistant Enterobacteriaceae (CRE) is vital for patient care in order to institute correct therapeutic options. Molecular techniques can easily differentiate classes but the need of the hour is a rapid practical phenotypic method which can differentiate KPCs and MBLs in Enterobacteriaceae. The present study detects and differentiates class A and B

carbapenemases among Enterobacteriaceae by simple phenotypic methods that uses both inhibitors EDTA and Phenyl Boronic Acid. Among 38 CRE isolated from 447 strains of Enterobacteriaceae 14 showed class B (MBL) and none showed class A (KPC) carbapenemases phenotypically. 19 were positive for MBL genes and none for KPC genes by multiplex PCR. To conclude, this phenotypic method is very helpful to detect carbapenemase production and provides a simple algorithm for the differentiation of Class A carbapenemases and MBL enzymes in Enterobacteriaceae in routine clinical microbiological laboratories.

Key words: carbapenemase resistant Enterobacteriaceae, phenotypic detection, MBL gene, KPC gene

1. INTRODUCTION

Carbapenems are commonly used to treat infections caused by multidrug-resistant Enterobacteriaceae (Gupta et al., 2012). The emergence of resistant to this group of drug is a major concern and has been noted all over the world (Tsakris et al., 2010). Resistance to carbapenems is mostly due to production of enzyme – Carbapenemases, which hydrolyse carbapenems and other beta-lactams. Other mechanisms are overproduction of ESBL or Amp C enzyme in organism with porin loss and decreased affinity of the penicillin binding proteins that constitute target proteins for carbapenems. Among these three mechanisms, carbapenemase production is the major cause and the rapid emergence and dissemination poses a considerable threat to clinical care and public health (Gupta et al., 2012; Datta Pet al., 2012).

Carbapenemases fall into Ambler classification- A, B and D. Class A includes enzymes such as KPC, IMI, SME etc which hydrolyse penicillin and cephalosporins more efficiently than carbapenems and are inhibited by boronic acid and less by clavulanate (Datta Pet al., 2012). Plasmid-encoded KPCs have emerged as the most clinically dominant member of the class. KPC producing Enterobacteriaceae were first reported in a clinical specimen from a patient in North Carolina in 2001 (Gupta et al., 2011).

Class B includes metallo- Betalactamases (MBL) namely VIM, IMP and SPM. These enzymes have a wide spectrum of activity against carbapenem, penicillin and extended spectrum cephalosporins and not on aztreonam. They are not hydrolysed by clavulanate but inhibited by chelating agents like EDTA. Class D carbapenemases belong to OXA family which hydrolyse carbapenems weakly and are poorly inhibited by clavulanate (Datta Pet al., 2012).

MBL and KPC enzymes hydrolyse almost all beta lactam antibiotics, and hence the phenotypic detection of each one of them can be masked by the expression of the other. Moreover they are transposon and /or integron encoded determinants that can easily disseminate to other enterobacterial strains (Tsakris et al., 2010). Presences of these enzymes are confirmed mainly by molecular methods, which are not available in every clinical laboratory. Practical and accurate phenotypic methods are needed to detect and differentiate these enzymes, helps clinicians to formulate the treatment and also prevention strategies.

2. MATERIALS AND METHODS

A total of 447 clinical isolates belonging to Enterobacteriaceae family collected over a period of one year were included in this study. All the strains were screened for the reduced susceptibility to carbapenems using Meropenem (10µg) and Ertapenem (10µg) discs by Kirby Bauer disc diffusion method according to CLSI guidelines and further confirmed using meropenem E strips. The strains which showed MIC <4µg for meropenem were considered as carbapenem resistant Enterobacteriaceae (CRE) (Clinical and Laboratory Standards Institute, Performance standards for antimicrobial susceptibility testing, 2012).

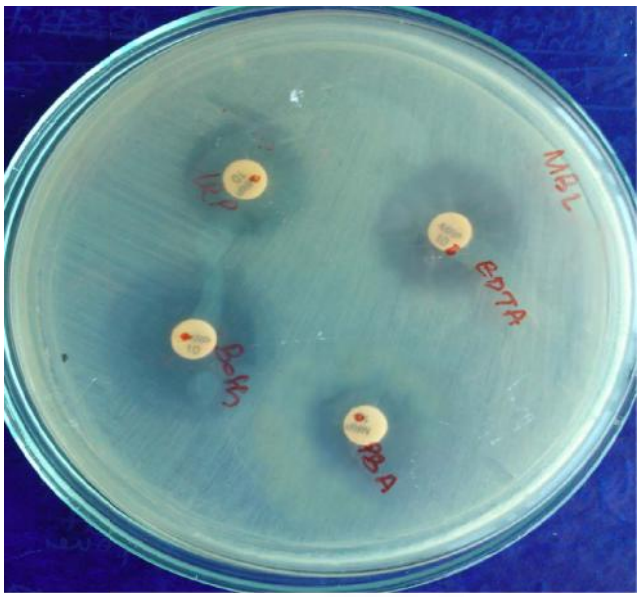
The identified strains of CRE were subjected to Modified Hodge (MHT) for the detection of carbapenemase production. Muller Hinton agar was lawn cultured with standard 0.5Mc Farland suspension of *E.coli* ATCC 25922. A single meropenem disc was placed on the plate (Gupta et al., 2011). 3-5 colonies of test organism grown overnight were inoculated in a straight line out from the edge of the disc with at least 20-25mm in length. Following incubation, enhanced growth showing clover leaf model was considered as carbapenemase producing and no enhanced growth as carbapenemase non producing strain respectively (Clinical and Laboratory Standards Institute, Performance standards for antimicrobial susceptibility testing, 2012).

Phenotypic detection of Class A carbapenemase

The stock solution was prepared by dissolving Phenylboronic acid (PBA) in DMSO at a concentration of 20mg/ml. from this solution, 20 µl was dispersed into commercially available meropenem discs. The discs were dried and used within 60min. The test strain was lawn cultured and discs placed on them and incubated. A zone diameter difference of >5mm with meropenem and PBA when compared to meropenem alone is considered to be KPC producer (Datta Pet al., 2012).

Phenotypic detection of Class B carbapenemase

The stock solution of EDTA was prepared by dissolving anhydrous EDTA in distilled water at a concentration of 0.1M. From this solution, 10µl (containing 292µg of EDTA) was dispensed onto meropenem discs. The discs were then dried and used within 60 minutes. The test organism was lawn cultured and the discs were placed on them. A zone diameter difference of >5mm of meropenem with EDTA compared to meropenem alone is said to be MBL producer (Figure 1). A zone diameter difference to both meropenem with EDTA and PBA were said to be both KPC and MBL producer (Datta Pet al., 2012).



Genotypic method

Genotypic detection of class A and class B carbapenemases were done by multiplex PCR. Oligonucleotides used are given in the table 1. The primers were reconstituted according to manufacturer's instructions. DNA was extracted with initial denaturation at 98^0C for 10minutes followed by thermocentrifugation for 10 minutes at 10,000rpm. The supernatant was collected and used for multiplex PCR amplification. The initial step of initial denaturation at 94^0C for 10 minutes was followed by annealing temperature at 55^0C for 2 minutes and extension temperature at 70^0C-72^0C for 1 minute. The amplicons were subjected to gel electrophoresis and the bands read in gel electrophoresis.

3. RESULTS

Out of 447 strains of Enterobacteriaceae isolated from various clinical samples over a period of one year, 38 (8.5%) isolates were confirmed as CRE which includes 12 *Escherichia coli*, 11 *Klebsiella pneumonia*, 1 *Klebsiella oxytoca*, 6 *Proteus mirabilis*, 2 *Proteus vulgaris*, 6 *Citrobacter freundii*. Of the 38 CRE isolates, 33 (86.84%) showed enhanced growth with clover leaf pattern indicating carbapenemase production. Table 2 shows the comparison of carbapenemase producing Enterobacteriaceae by MHT with PCR.

Phenotypic differentiation of class A and class B carbapenemases

On phenotypic differentiation, none of them showed to produce Class A (KPC) carbapenemase and 14 of them showed to be Class B (MBL) carbapenemases producers. Table 3 shows the comparison of phenotypic methods with PCR.

Genotypic detection of carbapenemases

38 isolates of Enterobacteriaceae species were subjected to genotypic detection of carbapenemase genes. 19 isolates of them showed the presence of carbapenemase genes in Multiplex PCR. One strain of *Proteus mirabilis* showed simultaneous co-existence of 2 genes NDM with IMP. No KPC genes were detected in our study. 19 of the CRE isolates showed class B carbapenemase (16 NDM, 2 IMP and 1both NDM & IMP). None of the CRE isolates showed class A (KPC) carbapenemase. Table 4 shows the distribution of carbapenemase genes in various isolates of CRE.

Table 1

Oligonucleotides used as primers for PCR in the study

Class	Genes		Gene sequence	Amplicon size
Class A	KPC	F	ATGTCACTGTATCGCCGTC	863bp
		R	AATCCCTCGAGCGCGAGT	
Class B	IMP	F	CCWAATITAAAAATYGAGAAGCTTG	522bp
		R	TGGCCAHGCTTCWAHATTTGCRTC	
	VIM	F	GTTTGGTCGCATATCGCAAC	382bp
		R	AATGCGCAGCACCAGGATAGAA	
	NDM	F	GGTGCATGCCCGGTGAAATC	660bp
		R	ATGCTGGCCTTGGGGAACG	

Table 2

Comparison of carbapenemase producing enterobacteriaceae by MHT with PCR

MHT	CARBAPENEMASE GENES BY PCR		TOTAL
	DETECTED	NOT DETECTED	
Positive	18	15	33
Negative	2	3	5
TOTAL	20	18	38

Table 3

Comparison of phenotypic methods with PCR

PHENOTYPIC DETECTION OF CLASS B CARBAPENEMASES	CLASS B CARBAPENEMASE GENES BY PCR		TOTAL
	DETECTED	NOT DETECTED	
Positive	11	4	14
Negative	8	15	24
TOTAL	19	19	38

Table 4

Distribution of carbapenemase genes in various isolates of CRE

Strain	NDM	IMP	NDM+ IMP	Total
Escherichia coli	5	-	-	5
Klebsiella pneumoniae	6	1	-	7
Citrobacter freundii	3	-	-	3
Proteus mirabilis	2	1	1	4
Total	16	2	1	19

4. DISCUSSION

Currently MBLs and KPC are considered a major threat in Enterobacteriaceae, representing a potential source of clinical failure in patients treated with almost all beta lactam agents (Tsakris et al., 2010). The prevalence of carbapenemase among gram negative bacilli varies greatly from country to country and among different institutions within the country (Doyle et al., 2012). The occurrence of CRE in our study is 8.8%. Priya D from Chandigarh, India noted 7.87% resistance rate. Gupta *et al* reported high prevalence of CRE varying from 17 to 22% (Datta Pet al., 2012).

Accurate detection of CRE by phenotypic and genotypic assays has an important clinical and epidemiological value. According to CLSI guidelines the modified Hodge test (MHT) should be used as a confirmatory test for carbapenemase production when the

initial screening tests are indicative (zone of inhibition by ertapenem or meropenem of ≤21mm in diameter in the disk diffusion assay or MICs of ertapenem, meropenem, and imipenem of 2, 2 to 4 and 2 to 4 µg/ml, respectively). The sensitivity and specificity of MHT in our study when compared with genotypic method are 90% & 16.7% respectively. 15 CRE isolates showed the production of carbapenemases by MHT, but were negative by genotypic method. This could be due to production of other types of carbapenemases like Class D, which was not included in this study. MHT is probably the best-known and most-used confirmatory assay, but its analytical performance is not optimal, also due to difficult interpretations. Moreover, MHT cannot discriminate among different types of carbapenemases and this may possibly contribute to the dissemination of additional resistance mechanisms among MBL and/or KPC producers (Balan et al., 2012).

None of the strains of CRE showed the production of class A carbapenemases (KPC) by inhibitor based phenotypic method which 100% specificity with genotypic method. We found the sensitivity and specificity of EDTA based phenotypic detection of MBL carbapenemases is 57.9% and 78.94%. A study conducted by Xia Yun concluded the sensitivity and specificity of the EDTA-disc synergy tests for MBLs detection were 85.7% and 100%, respectively (Xia et al., 2013). In another study conducted in Australia, the overall sensitivity and specificity of the phenotypic MBL detection method were 100% and 98%, respectively (Franklin et al., 2006). The phenotypic detection of Class A & B can thus be considered to be more specific. Samples with positive results can be detected genotypically for further confirmation.

To conclude, microbiologic excellence is a timely detection of resistant pathogens and can help formulate effective prevention and infection control strategies and help make better patient outcomes possible. This phenotypic method is very helpful to detect carbapenemase production and provides a simple algorithm for the differentiation of Class A carbapenemases and MBL enzymes in Enterobacteriaceae in routine clinical microbiological laboratories.

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