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Identification of Class A, B and D Carbapenemases by Phenotypic and Molecular methods in organisms of the family Enterobacteriaceae

Dr. Sanjeev Kumar Assistant Professor, Department of Microbiology, PIMS, Udaipur, Rajasthan. 08440086034.

Dr. S.K. Mehra Ex-Professor & HOD, Department of Microbiology, GMCH, Udaipur, Rajasthan

ABSTRACT Detection of carbapenemases in particular members of the Enterobacteriaceae, is of utmost importance for the management of infected or colonized patients. Antimicrobial susceptibility was determined by disc diffusion method, results were interpreted according to CLSI guidelines. Phenotypic confirmation was done by modified Hodge test and Combined disc test. After phenotypic confirmation, 20 randomly selected isolates were further studied for the presence of carbapenem resistance (CRE) genes. By the molecular method (PCR), 17 isolates were positive for carbapenemase production and 3 isolates were showed non-specific PCR amplicons. All the 20 isolates were positive by combined disc method and only 8 isolates were positive by MHT. The sensitivity and specificity of the combined disc test was 100% and 66.67%, whereas modified Hodge test was 37.5% and 33.33% respectively, in comparison with the gold standard PCR method. Before performing such an expensive technique (PCR), we have to confirm by phenotypic methods.

KEYWORDS : Carbapenemases, Polymerase chain reaction (PCR), Modified Hodge test, combined disc test.

INTRODUCTION:

Carbapenems are commonly used to treat infections caused by multidrug-resistant (MDR) Enterobacteriaceae. The spread of carbapenemases in Enterobacteriaceae is among the most important issues in the antimicrobial resistance. Carbapenemases, the enzymes conferring hydrolysis of carbapenems, are diverse and include representative of β-lactamase molecular classes A, B, and D(OXA). In class A carbapenemases, KPC variants are now spreading rapidly.[17] KPC-carbapenemases are capable of hydrolyzing carbapenems, penicillins, cephalosporins and aztreonam and are inhibited by clavulanic acid. Combined-disc tests based on the use of a KPC inhibitor, boronic acid, have also recently been described as promising methods for the detection of organisms with KPC enzymes.[12,15,16] The class B metalloenzymes (IMP, VIM etc.,) have a broader substrate of hydrolysis ranging to penicillins, cephalosporins, cephamycins, oxacephamycins and carbapenems but not monobactams. Thus, the detection of MBL's is based on the enzyme's zinc dependence by using as inhibitors chelating agents such as EDTA.[3] The first identified OXA-48 producers were from a K. pnemoniae strain isolated in Turkey in 2003.[13] OXA-48/OXA-181 are peculiar because they weakly hydrolyze carbapenems and broad-spectrum cephalosporins such as ceftazidime and aztreonam.[13,2] Their activity is not inhibited by EDTA or clavulanic acid, although reported in various enterobacterial species.[8]

A number of simple phenotypic tests most of them in the disk diffusion format; have been described and evaluated as methodologies for the specific detection of carbapenemase producing organisms. The modified Hodge test has been extensively used as a general phenotypic method for the detection of carbapenemase activity.[10,12] Several inhibitor-based tests have been developed for the detection of MBL is based on the enzyme's zinc dependence by using as EDTA.[10,18] Combined disc tests based on the use of meropenem alone and with EDTA, phenyl boronic acid (PBA), EDTA plus PBA, have also recently been describes as promising methods for the detection of MBL, KPC and both MBL&KPC.[9]

Molecular techniques are nowadays considered the gold standard for the optimal identification of different types of carbapenemase producing Enterobacteriaceae.[11] In spite of increasing reports on these carbapenem resistance genes from various countries, such reports are scattered from Rajasthan, India. Therefore, the present study was performed to look for the molecular detection of blaKPC, blaIMP, blaVIM and blaOXA, genes responsible for carbapenem resistance in Enterobacteriaceae isolates that were positive by phenotypic confirmatory test (Combined disc test and modified Hodge test [MHT]).

MATERIALAND METHODS:

The present study was performed in the department of Microbiology of Pacific Medical College and Hospital (PMCH) and the clinical isolates were collected from Geetanjali Medical College and Hospital as well as from PMCH on routine samples subjected to culture and antibiotics susceptibility during December 2012 to December 2014. Antimicro bial susceptibility was determined, including meropenem, and the zone diameter of meropenem ≤21 mm was included in the study for further confirmation according to CLSI guidelines.[4] Phenotypic confirmation was done by modified Hodge test and Combined disc test.[9] After phenotypic confirmation, 20 randomly selected isolates were further studied for the presence of carbapenem-resistance genes (blaIIMP, blaVIM, blaOXA and blaKPC).

Modified hodge test (MHT):

The test is performed by inoculating a Muller Hinton Agar plate with a 1:10 dilution of a 0.5McF suspension of susceptible strain E. coli ATCC 25922 as describes in the routine disk diffusion CLSI procedure. Then the plate is allowed to dry 3-10 minutes. Next, one carbapenem disk was placed on the plate. Subsequently, by using a 10ul loop, 3-5 colonies of the test and QC organisms, grown overnight on an agar plate, are inoculated onto the plate in a straight line from the edge of the disk. There after incubated for 18-24 hours at 35–370C and noted the results (CLSI, 2010).

Interpretation

Alteration in the shape (indentation) of the zones of inhibition around the test organism is considered indicative of the presence of a carbapenemase.

Phenotypic method for differentiation of KPC and MBL:

Phenyl boronic acid (PBA), EDTA or both along with meropenem disc were used for detection of KPC and MBL, respectively. On Muller Hinton agar plate inoculated with test strain, four discs of meropenem were used. One disc of meropenem using without any inhibitor, one disc have PBA only, one disc have EDTA only and fourth disc of meropenem have both PBA and EDTA. The agar plates were incubated at 370C overnight and the diameter of the growth inhibitory zone around these meropenem discs with inhibitor added were compared with that around the plain meropenem disc (Tsakris et al., 2010).

Interpretation:

The isolates are considered KPC producing when the growth inhibitory zone diameter around the meropenem disc with PBA and the meropenem disc with both PBA and EDTA were increased ≥5mm was compared with the growth-inhibitory zone diameter around the disc containing meropenem alone.

The isolate are considered MBL producing when the growth inhibitory zone diameter around the meropenem disc with EDTA and the meropenem disc with both PBA and EDTA were increased ≥5mm was compared with the growth-inhibitory zone diameter around the disc containing meropenem alone.

The isolate are considered producing both KPC and MBL enzyme when the growth- inhibitory zone diameter around the meropenem disc with both PBA and EDTA were increased ≥5mm was compared with the growth-inhibitory zone diameter around the disc containing meropenem alone while the growth-inhibitory zone diameters around the meropenem disc with PBA and the meropenem disc with EDTA were increased <5mm was compared with the growth- inhibitory zone diameter around the disc containing meropenem alone.

The isolates are considered negative for MBL and KPC production, when none of the three combined-disc tests were positive (Tsakris et al., 2010).

Detection of bla genes (blaIMP, blaVIM, blaOXA and blaKPC) responsible of carbapenem resistance:

The primer sets used for detection of carbapenem-resistance bla genes are shown in Table 1. The primers for detection of β-lactamase (bla) genes were synthesized and all the procedures for detection of bla genes were done at Chromous Biotech, Banglore, India.

Genomic DNA was isolated following the Chromous RKN13 protocol. Primers were designed for amplification & PCR amplification was performed by Thermal cycler (ABI2720) and all PCR reagents were of Chromous make.

Table 1: List of the PCR-primers used

Targets	Primer sequence (5' to 3')
blaKPC	FP: CTTGCTGCCGCTGTGCTG RP: GCAGGTTCCGGTTTTGTCTC
blaIMP	FP: GAATAGRRTGGCTTAAYTCTC RP: CCAAACYACTASGTTATC
blaVIM	FP: GTTTGGTCGCATATCGCAAC RP: AATGCGCAGCACCAGGATAG
blaOXA	FP: ATGGCAATCCGAATCTTC RP: TTATCGCGCAGCGTCCGAG

RESULT & DISCUSSION:

Among the 157 carbapenem resistant Enterobacteriaceae detected by phenotypic confirmatory tests (modified Hodge test (MHT) and Combined disc test), 20 randomly selected isolates were studied for molecular characterization of blaKPC, blaIMP, blaVIM and blaOXA genes at Chromous Biotech, Bangalore, India. From these 20 Enterobacteriaceae isolates, 10 were E.coli and 10 K.pneumoniae. We noticed that, only a few reports characterizing the carbapenem-resistance genes in Indian bacterial population at a molecular level.[1,5] Another observation is that the molecular reports on occurrence of carbapenem bla genes in Rajasthan, India, are also limited.

Table 2 shows the molecular detection of carbapenemase production in 20 carbapenem resistant isolates in comparison with combined disc test and modified Hodge test (MHT). By the molecular method such as polymerase chain reaction (PCR), 17 isolates were positive for carbapenemase production and 3 isolates were showed non-specific PCR amplicons[Fig 1]. Whereas all the 20 isolates were positive by combined disc method and only 8 isolates were positive by MHT. Moreover 4 isolates were positive for multiple genes by PCR, of them 3 isolates were also showed positive for multiple carbapenemase production by combined disc method. The sensitivity and specificity of the combined disc test was 100% and 66.67%, whereas modified Hodge test was 37.5% and 33.33% respectively, in comparison with the gold standard PCR method.

Molecular techniques, nowadays considered as the gold standard for the optimal identification of different types of carbapenemase-producing genes in *Enterobacteriaceae*, although it only detects a pre-specified set of known genes and primarily we have to design the primers for the interested genes. However the primers are commercially available in the market. Here we studied randomly selected 20 clinical isolates of carbapenemase resistant *Enteroba cteriaceae* species by PCR for detection of blaKPC, blaVIM, blaIMP and blaOXA genes, which are currently the most prevalent carbapenemases.[14]

Table 2: Detection of Carbapenemases by Combined disk test and Modified Hodge Test in comparison with Polymerase Chain Reaction (PCR)

S.No.	Organism	*PCR	$CDT	#MHT
1	*E.coli*	blaOXA	KPC	Positive
2	*E.coli*	blaIMP	MBL	Negative
3	*E.coli*	blaIMP	MBL	Negative
4	*E.coli*	blaKPC	KPC	Positive
5	*E.coli*	blaIMP	MBL	Negative
6	*E.coli*	blaIMP	MBL	Negative
7	*E.coli*	blaIMP	MBL	Positive
8	*E.coli*	blaKPC	KPC	Positive
9	*E.coli*	blaIMP	MBL	Negative
10	*E.coli*	blaIMP	MBL	Negative
11	*K.pneumoniae*	blaOXA, blaVIM, blaKPC	KPC+MBL	Positive
12	*K.pneumoniae*	blaKPC, blaIMP	KPC+MBL	Negative
13	*K.pneumoniae*	blaKPC, blaIMP	KPC+MBL	Negative
14	*K.pneumoniae*	Negative	Negative for KPC&MBL	Positive
15	*K.pneumoniae*	blaIMP	MBL	Negative
16	*K.pneumoniae*	Negative	KPC	Negative
17	*K.pneumoniae*	blaIMP	KPC	Negative
18	*K.pneumoniae*	blaIMP	MBL	Negative
19	*K.pneumoniae*	Negative	Negative for KPC&MBL	Positive
20	*K.pneumoniae*	blaVIM, blaIMP, balaKPC	KPC	Positive

PCR-polymerase chain reaction; $CDT-combined disc test; #MHT-modified Hodge test; bla-β-lactamase; OXA-oxacillinases; IMP-imipenemase; VIM-verona integron-encoded metallo β-lactamase; KPC-Klebsiella pneumoniae carbapenemases;

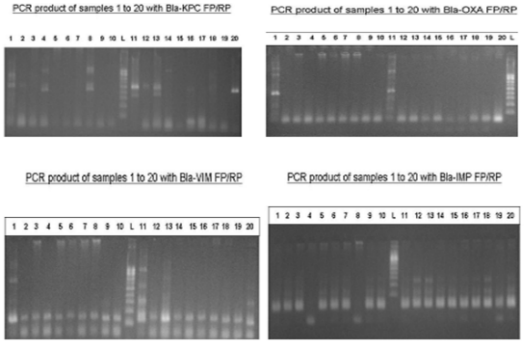

Fig 1: Results of blaKPC, blaOXA, blaVIM and blaIMP after PCR amplification.

In our study, we detected bla-IMP gene was the most common followed by bla-KPC, bla-VIM and bla-OXA. Among the 20 isolates, 17(85%) were positive for different carbapenemase production and 15(75%) isolates were correlates with phenotypic confirmatory test such as combined disc test and only 6(35.29%) isolates were positive by modified Hodge test (MHT). Previous studies conducted by Girlich et al[6], Giske et al[7], Pasteran et al.[12] and Tsakris et al.[16] reported the modified Hodge test and carbapenemease inhibition tests with boronic acid or EDTA/DPA, do not provide information on the carbapenemase gene and cannot differentiate OXA-48 producing isolates from ESBL and/or AmpC producing isolates with reduced permeability. In the present study, we also cannot differentiate the OXA producing isolates by phenotypic method.

There are several pros and cons using either phenotypic or molecular methods for the detection of resistance mechanisms in Enteroba cteriaceae. Phenotypic tests require bacteria in pure culture from a clinical sample that requires 24-48 hours to obtain a final result. Molecular techniques on the other hand, can be performed directly with clinical specimens reducing significantly the procedure time. The detection of low-level resistance is by definition problematic using phenotypic test thus interpretation problems may appear. In such cases, molecular techniques are an option for clarifying the possible involvement of any known resistance mechanism.

Moreover, genetic detection gives a precise answer for the presence or absence of specific resistance determinants within a study isolate, whereas this is not possible with the phenotypic tests which provide only general information about the resistance mechanisms involved. However, genetic assays also some major limitations; it is possible to screen exclusively for known mechanisms and for one gene at the time (unless a multiplex PCR assay can be applied) and their cost is high and becomes higher when screening for multiple resistance determinants. In addition need of expensive equipment and reagents and expert personnel, which are not always available to the diagnostic laboratory.

CONCLUSION:

Forever, molecular techniques considered as the gold standard for the optimal identification of different types of carbapenemase-producing genes in Enterobacteriacea. Before performing such an expensive technique (PCR), we have to confirm by phenotypic methods. Our results demonstrate that in regions where prevalence data suggest dissemination of MBL and KPC carbapenemases, boronic acid compounds are useful tools in the clinical laboratory not only for the detection of KPCs but also in combination with EDTA for the identification of possibly co-produced MBLs, by means of a combined-disc method using meropenem alone and with PBA, EDTA, both PBA plus EDTA. Also, since these enzymes can easily be transmitted via transposon and or integron, there is possibility of widespread dissemination among susceptible gram negative bacterial isolates in the hospital. The need of the hour is microbiological laboratory that detects CRE accurately and timely for better patient outcomes.

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