Original article:

Differentiation of Carbapenemase producing Enterobacteriaceae by Triple disc Test

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Abstract:

Background: Carbapenems are a class of beta-lactam antibiotics with a broad spectrum of antibacterial activity. They are often considered as last resort antibiotics in the treatment of infections due to multidrug-resistant organisms. Carbapenemase-producing *Enterobacteriaceae* (CPE) have been reported worldwide.

Objective: Present study was carried out to differentiate Carbapenemase producing Enterobacteriaceae by Triple Disc Test (using meropenem, phenyl boronic acid and EDTA Discs) among various clinical isolates received in Department of Microbiology, SMS Medical College & attached Hospital, Jaipur.

Methods: Carbapenemase producing meropenem resistant Enterobacteriaceae species isolated from clinical samples were included in the study. The study was designed for differentiation of KPC and MBL enzymes by using Triple Disk Tests consisting of meropenem alone and with phenylboronic acid (PBA), EDTA, or both PBA and EDTA Augmentation of the zone of inhibition by ≥5 mm was considered a positive combined-disc test result.

Results: A total of 347 phenotypically confirmed carbapenemase-positive Enterobacteriaceae clinical isolates were examined. Out of these 194 strains were KPC producer, 102 strains were MBL producer and 51 strains were both KPC and MBL producers.

Conclusions: This phenotypic method is very helpful to detect carbapenemase production and provides a simple algorithm for the differentiation of KPC and MBL enzymes, and guides in empirical treatment of patients especially in regions where KPC- and MBL-possessing Enterobacteriaceae are highly prevalent.

Key words: Triple-disc test, *Klebsiella pneumonia* , carbapenemase

Introduction

Enterobacteriaceae is a family of gram-negative, rod-shaped facultative anaerobic bacteria. *Escherichia coli*, *Klebsiella pneumonia*e and related organisms commonly live in the enteric tract. Critically ill patients are vulnerable to infection by these organisms because of breaches in the normal skin barrier or insertion of invasive devices.[1,]

Carbapenems are a class of beta-lactam antibiotics with a broad spectrum of antibacterial activity.[2] They have a structure that renders them highly resistant to most beta-lactamases. They are often considered as last resort antibiotics in the treatment of infections due to multidrug-resistant organisms. However, during the last decade carbapenem resistance has been increasingly reported among Enterobacteriaceae due to production of carbapenemase.[3]

Carbapenemase are a diverse group of β-lactamases, divided into different classes, depending on the structure of the enzyme and the mechanism by which

they hydrolyze the β-Lactam ring. Carbapenemases identified in *Enterobacteriaceae* mainly belongs to 3 classes of β-lactamases: the Ambler class A, B, and D. [4] The most clinically significant Ambler class A carbapenemases are of the *Klebsiella pneumoniae* carbapenemases [KPC] type. [5] They hydrolyse all β -lactams, and their activity is inhibited by boronic acid and, partially by clavulanic acid and tazobactam. The first KPC producer (KPC-2 in *K. pneumoniae*) was detected in North-Carolina, US, in 1996 and has since spread worldwide.[6] Class B enzymes are metallo β lactamases (MBLs). These enzymes exhibit a broad spectrum of hydrolytic activity, including all penicillins, cephalosporins, and carbapenems, sparing only the monobactam aztreonam. [5, 7] Their activity is not inhibited by commercially available β lactamase inhibitors (clavulanic acid, tazobactam, or sulbactam) but they are inhibited by chelating agents such as EDTA. The most important MBLs include the VIM and IMP VIM, SPM and NDM types [5,7]. The Ambler class D enzymes with carbapenemase activity in Enterobacteriaceae are mostly OXA-48 and OXA-181. [5] They have a atypical hydrolysis profile, sparing ceftazidime, hydrolysing cefotaxime at a very low level, and are weekly inhibited by clavulanic acid– tazobactam.

Detection of carbapenemase producers in Entero-bacteriaceae is important, as carbapenemases are also associated with many other resistance determinants, giving rise to multidrug resistance and even pandrug resistance. It is also important for patient care as to start appropriate therapeutic options. Another important epidemiological implication is that both MBLs and KPCs are spread by transposon and/or integron-encoded determinants that can also carry non-β-lactam resistance determinants, which can disseminate to other enterobacterial strains.[8]

The Enterobacteriaceae isolates that harbor both MBL and KPC carbapenemases are increasingly recovered from clinical specimens, and this has led to difficulty differentiating and identifying these enzymes. This study reports on the implementation of a phenotypic method that uses both the inhibitors EDTA and boronic acid to differentiate class A and B carbapenemases Molecular techniques are the gold standard method for the detection of carbapenemase production in Enterobacteriaceae but it is not suitable for daily testing in resource limited clinical laboratories due to the cost and inconvenience.[7] Therefore it is important to use a simple, rapid and cost effective phenotypic method to differentiate carbapenemase production in Enterobacteriaceae.

Materials and methods:

Present study was carried out in the Department of Microbiology & Immunology, Sawai Man Singh Medical College and Hospitals, Jaipur (Rajasthan) during October 2011 and September 2012. Clinical samples were received for culture and sensitivity testing from various medical and surgical wards, intensive care units (ICUs) and outdoor patients department (OPDs). Organisms identified as member of enterobacteriaceae by standard biochemical reactions.[9] Antimicrobial sensitivity testing of isolated microorganisms was performed by Kirby Bauer disc diffusion method as per CLSI guidelines.[10] A total of 347 carbapenemase producing meropenem resistant strains isolated from various clinical samples were included in the study and tested for differentiation of carbapenemase produced.

For Carbapenemase production The Modified Hodge Test (MHT) as originally described by the Centre for

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Disease Control (CDC) was used.[10] Differentiation of Carbapenemase was done by Triple Disc Test using meropenem phenylboronic acid (PBA), and EDTA. [11]

Interpretation

Production of KPC was considered when the growth-inhibitory zone diameter around the meropenem disc with PBA and the meropenem disc with both PBA and EDTA was increased ≥5 mm compared with the growth-inhibitory zone diameter around the disc containing meropenem alone.

Production of MBL was considered when the growth-inhibitory zone diameter around the meropenem disc with EDTA and the meropenem disc with both PBA and EDTA was increased ≥5 mm compared with the growth-inhibitory zone diameter around the disc containing meropenem alone.

Production of both KPC and MBL enzymes was considered when the growth-inhibitory zone diameter around the meropenem disc with both PBA and EDTA was increased ≥5 mm 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 ≤5 mm compared with the growth-inhibitory zone diameter around the disc containing meropenem alone.

When none of the three combined-disc tests was positive, the isolate was considered negative for MBL and KPC carbapenemase production.

Results

The study was conducted on 347 carbapenamase producing meropenem resistant strains isolated from various clinical samples. All the isolates were tested for carbapenemase production by MHT. Maximum number of carbapenemase producing strains were isolated from pus (45.24%) followed by tracheal secretion (32.56%). (Table-I)

Enterobacter species were the most common carbapenemase producing species isolated from 43.52 % of samples followed by *Escherichia coli and Klebsiella species* 20.75% and 20.46% respectively.

In present study out of 347 Carbapenemase producing Enterobacteriaceae 194 KPC producers belonged to nine different enterobacterial species, 102 MBL producers to seven enterobacterial species, while all 51 isolates that co-produced KPC and MBL carbapenemases were of five enterobacterial species. (Table-II)

Table I: Isolation of Carbapenemase producing Enterobacteriaceae strains from various clinical samples

NATURE OF SAMPLE	NUMBER	PERCENTAGE
Pus	157	45.24%
Tracheal secretion	113	32.56%
Sputum	33	9.51%
Body fluids	18	5.19%
Others	14	4.03%
Tissue	7	2.02%
Wound swab	5	1.44%
TOTAL	347	100%

Table II: Differentiation of Carbapenemase producing Enterobacteriaceae

Organisms	Number of strains isolated	KPC	Percentage	MBL	Percentage	Both	Percentage
Escherichia coli	72	45	62.5%	20	27.7%	7	9.7%
Klebsiella species	71	44	61.97%	17	23.94%	10	14.08%
Enterobacter cloacae	77	43	55.84%	18	23.37%	13	16.88%
Enterobacter aerogenes	74	34	45.94%	29	39.18%	14	18.91%
Citrobacter species	42	19	45.23%	16	38.09%	7	16.66%
Proteus vulgaris	5	4	80%	1	20%	0	0%
Arizona species	2	2	100%	0	0%	0	0%
Proteus mirabilis	2	2	100%	0	0%	0	0%
Proteus morgani	1	1	100%	0	0%	0	0%
Proteus species	1	0	0%	1	100%	0	0%
TOTAL	347	194	55.9%	102	29.39%	51	14.69%

Discussion

The implementation of a simple and accurate laboratory method to detect carbapenemase production in Enterobacteriaceae is useful, particularly in countries where multi drug resistant strains are increasingly reported.[3, 5]

The prevalence of Carbapenemase producing Enterobacteriaceae varies from place to place and with geographical regions. The prevalence of Carbapenemase Producing Enterobacteriaceae reported from India range from 7 to 51 %. [12, 13]

In present study 347 MHT positive meropenem resistant Enterobacteriaceae strains were included for differentiation of carbapenemase production in Enterobacteriaceae. It is suggested that the modified Hodge test should be used as a confirmatory test for carbapenemase production when the initial screening tests are indicative (carbapenem MICs>.1 mg/L)[14] However, this test is often difficult to interpret, and is only indicative of enzymatic activity of carbapenemase and cannot differentiate class A carbapenemases from class B MBLs. [11]

In our study out of 347 Carbapenemase producing Enterobacteriaceae isolates 55.9% were KPC producers, 29.39% were MBL producers and 14.79% were both KPC and MBL producers. In a study conducted by Baraniak, J et al [15] they reported 57.9% were KPC producer and, and 43.4% were MBL producers. It is comparable to that of our study. In a study conducted by Tsakris A et al[11] out of 141 carbapenemase-positive Enterobacteriaceae clinical isolates 44.68% were KPC producers, 33.33% were MBL producers, and 21.98% were KPC and MBL producers. They reported Triple Disc Test 100% sensitive for detection of KPC & MBL comparing it with molecular methods.

The study was designed to differentiate Carbapenemase produced by enterobacteral species. Most of the studies in our region mainly stressed to diagnose MBLs and few have isolated KPCs[16-18]. We differentiate both the Carbapenemase produced by different enterobacteral species. *Escherichia coli* 62.5% of the strains were KPC producers while MBL was produced by 27.7% of strains. Similarly in *Klebsiella species* 61.97% were KPC producers while MBL was produced by 23.94%. It was noted that in enterobacteral species KPC producers were more than MBL producers. The MBLs are mainly responsible for carbapenemase production in pseudomonas species.

We conclude that Triple Disc Test for KPC and MBL production is simple to perform and materials used are cost-effective, non toxic and easily available which makes it highly pertinent as screening test in routine clinical laboratory. With this it also guides the clinician regarding empirical treatment

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