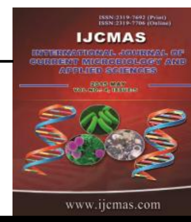
Int.J.Curr.Microbiol.App.Sci **(2015) 4(5): 255-261**



International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 5 (2015) pp. 255-261**
http://www.ijcmas.com

Original Research Article

Performance of Modified Hodge Test and Combined Disc Test for Detection of Carbapenemases in Clinical Isolates of Enterobacteriaceae

Sanjeev Kumar* **and S.K.Mehra**

Department of Microbiology, PMCH, Udaipur, Raj, India

**Corresponding author*

Keywords

KPC,
MBL,
modified Hodge test,
Combined-disc test.

A B S T R A C T

Carbapenemase producers are increasingly reported worldwide in Enterobacteriaceae. The increasing frequency of class A (KPC) and class B metallo-β-lactamases (MBLs) among Enterobacteriaceae, in addition their possible co-production makes their early detection and differentiation helps to the patients as well as clinicians. The aim of the present study is to evaluate the performance of modified Hodge test and combined disc test for detection and differentiation of carbapenemase production. The study included 50 carbapenem resistant clinical isolates of Enterobacteriaceae. All the isolates were identified by standard microbiological procedures and antibiotic sensitivity was performed according to CLSI guidelines. All the isolates were tested for carbapenemase production by modified Hodge test (MHT) and combined disc test. Among the 50 carbapenem resistant isolates, only 17 isolates were positive and 33 isolates were negative by MHT. Of these 50 carbapenem resistant clinical isolates of Enterobacteriaceae, 46 isolates were producing KPC, MBL and KPC+MBL enzymes. Four isolates were negative for KPC and MBL by combined disc test. We conclude that modified Hodge test is not a useful method for differentiation of carbapenemase production. Combined disc method is useful to detect carbapenemase production and helpful for the differentiation of KPC and MBL enzymes, especially regions where these enzymes are highly prevalent among the isolates of Enterobacteriaceae.

Introduction

Carbapenems are often the antimicrobials of last resort to treat multi-drug resistant gram negative bacilli. Although during the last few years carbapenem resistant Enterobacteriaceae (CRE) has been increasingly reported. Their identification is of primary importance because carbapenemase producers are resistant to almost all β-lactams and also other class of antibiotics. Mainly, three types of carbapenemases are now commonly identified in Enterobacteriaceae. They are the Ambler class A (KPC), class B (MBL) and class D (OXA) types (Ambler *et al.,* 1991; Nordman *et al.,* 2011; Walsh *et al.,* 2005). They have become widespread in

several regions of North and South America as well as in Israel, China, Greece (Bratu *et al.,* 2005; Cai *et al.,* 2008; Kitchel *et al.,* 2009; Pournara *et al.,* 2009) and India (Datta *et al.,* 2012). The Enterobacteriaceae isolates that harbour both MBL and KPC carbapenemases are increasingly recovered from clinical specimens and this has led to difficulty to differentiating and identifying these enzymes (Bansal *et al.,* 2013). Various techniques can be used for detecting production of carbapenemase enzymes, like phenotypic to advanced molecular techniques. 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 (MHT) has been widely used as a general phenotypic method for the detection of carbapenemase activity (Bansal *et al.,* 2013), and it is the only recommended method by the CLSI, for carbapenemase detection (CLSI, 2011).

It is based on the inactivation of a carbapenem by carbapenemase producing strains that enables a carbapenem susceptible indicator strain to extend growth towards a carbapenem disk, along the streak of inoculums of the tested strain. The assay is useful for the detection of a carbapenemase-mediated mechanism of resistance to carbapenems but does not provide information regarding the type of carbapenemase involved. Moreover, there have been reports of false-positive results, mostly generated by CTX-M-producing strains with reduced outer membrane permeability, and some investigators have raised the problem of difficulties in the interpretation of the clover leaf test for weak carbapenemase producers, particularly for MBL production in *Enterobacteriaceae.*[12] Several inhibitor based methods have been developed for the detection of MBLs and KPCs (Pasteran *et al.,* 2009; Franklin *et al.,* 2006; Tsakris *et al.,* 2009). Therefore, practical and accurate phenotypic approaches are urgently needed to differentiate the horizontally acquired mechanisms or reduced susceptibility to carbapenems among Enterobacteriaceae in the clinical laboratory. The aim of this study was to evaluate the phenotypic methods such as modified Hodge test and combined disc test for detection of carbapenemases in clinical isolates of Enterobacteriaceae.

Materials and Methods

Bacterial strains

The study includes non-duplicate isolates of *Enterobacteriaceae* are obtaining from different clinical samples. The clinical samples included in the study are pus, wound swabs, body fluids, sputum, throat swab and endotracheal secretions were submitted to the microbiology laboratory. The standard microbiological techniques used for the isolation and identification of the isolates. The *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC BAA-1705 strains were used for quality control.

Antimicrobial susceptibility testing and phenotypic screening

The susceptibility test was carried out by Kirby-Bauer disc diffusion technique following the CLSI guidelines and interpretative criteria. Carbapenemase production was screened by disc diffusion; all the isolates with a reduced susceptibility to meropenem (diameter of zones of inhibition, ≤ 21mm) were considered as screening positive and further tested by phenotypic confirmatory methods.

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 35–37^0 C and noted the results (CLSI, 2011).

Interpretation

Alteration in the shape (indentation) of the zones of inhibition around the test organism is considered indicative of the presence of a carbapenemase (Fig 1).

Phenotypic method for differentiation of KPC and MBL

Phenylboronic acid (PBA), EDTA or both along with meropenem disc will be using for detection of KPC and MBL, respectively. The stock solution of PBA in the concentration of 20 mg/ml will be prepared by dissolving PBA in DMSO. Twenty microliters (400 ug of PBA) from this solution will be dispensed onto meropenem discs. The stock solution of EDTA will be prepared by dissolving anhydrous EDTA in distilled water at concentration of 0.1M. Ten microliters (292 ug of EDTA) from this solution will dispense onto meropenem discs. The meropenem discs with inhibitor added will dried and using within 60 minutes.

On Muller Hinton agar plate inoculated with test strain, four discs of meropenem will be using. 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 will be incubated at 37^0C overnight and the diameter of the growth inhibitory zone around these meropenem discs with inhibitor added will compare with that around the plain meropenem disc (Tsakris *et al.,* 2010).

Interpretation

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

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

The isolate will considered producing both KPC and MBL enzyme when the growth-inhibitory zone diameter around the meropenem disc with both PBA and EDTA will increased ≥5mm will 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 will compared with the growth-inhibitory zone diameter around the disc containing meropenem alone (Fig. 1).

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

Result and Discussion

From the 746 clinical isolates of Enterobacteriaceae, 50 (6.7%) isolates were showed reduced susceptibility to meropenem. Among the 50 isolates of carbapenem resistant Enterobacteriaceae, 28(56%) *E. coli*, 18(36%) *Klebsiella*, 02(4%) *Enterobacter* and 02(4%) *Citrobacter*. All the isolates were tested for carbapenemase by modified Hodge test (MHT) and combined disc test. Among the 50 carbapenem resistant isolates, only 17(34%) isolates were positive and 33(66%) isolates were negative by MHT. Of these 50 carbapenem resistant clinical isolates of Enterobacteriaceae, 46(92%) isolates were producing KPC, MBL and KPC+MBL enzymes. Four isolates were negative for KPC, MBL and both by combined disc test. Among the 46 carbapenemase producing isolates, 09(19.5%) were produced KPC, 28(60.8%) isolates produced MBL and 09(19.5%) isolates produced both KPC+MBL (Table 1).

Enterobacteriaceae majorly contribute to the intrinsic human gut flora. They are also capable of colonizing the gut of patients and spreading through the community via the faeco-oral route. Hence the spread of carbapenemase resistant *Enterobacteriaceae* is deeply alarming in a country such as India with a reservoir of more than 1.4 billion people (Nordman *et al.,* 2011; Paterson, 2006).

The evaluation of a simple and accurate laboratory method to detect carbapenemase production in clinical isolates of Enterobacteriaceae is helpful, particularly in countries where multi-drug resistant strains are increasingly reported (Nordman *et al.,* 2011; Schwaber and Carmeli, 2008). Currently MBLs and KPCs are considered a major threat in Enterobacteriaceae, representing a potential source of clinical failure in patients treated with almost all β-lactam antibiotics (Queenan and Bush, 2007; Nordman *et al.,* 2009). In the present study out of 50 crabapenem resistant Enterobacteriaceae, 09(19.5%) were produced KPC, 28(60.8%) isolates produced MBL and 09(19.5%) isolates produced both KPC+MBL. In a study conducted by Bansal *et al.* (2013) and Baraniak *et al.* (2013) they reported KPC producers were more common than the MBL producers, while in our study MBL producers were common.

Although MBL and KPC co-production has been reported in *K. pneumoniae* (Giakkoupi *et al.,* 2009) and currently is widely detected in Enterobacteriaceae. Moreover, KPC and MBL genes are often co-transferred with plasmid-mediated ESBL, fluoroquinolone and aminoglycoside resistant genes. In our study co-production of MBL and KPC (19.5%), which was similar to the study conducted by Tsakris *et al.* (2010) they have reported co-production of MBL and KPC was 21.98%.

Therefore, the simple and reliable phenotypic detection of MBL and KPC carbapenemases is important for clinical and epidemiological purposes, adding to studies on the preliminary characterization of the antimicrobial resistance mechanisms. In this study, we have observed the occurrence of false results of carbapenemase production by the modified Hodge test (MHT) among isolates in which carbapenem reduced susceptibility or resistance was detected. Among the 50 carbapenem resistant isolates, only 17(34%) isolates were positive and 33(66%) isolates were negative by MHT. In contrast to the present study Deshpande *et al.* (2010) reported 91.6% were MHT positive. Furthermore, high false-positive rates have been reported. These are mainly caused by CTX-M type ESBLs and to a

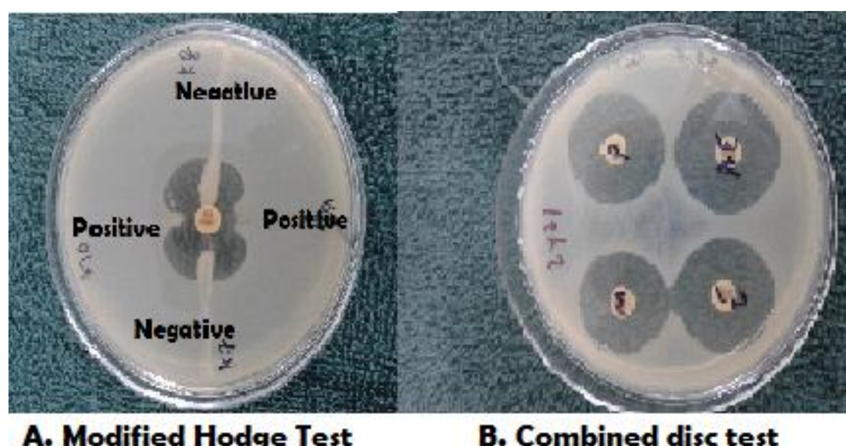
lesser extent, AmpC hyperproduction and ESBL production coupled with porin loss or porin mutations (Pasteran *et al.,* 2010; Carvalhaes, 2010). Apart from being technically demanding and time consuming, disadvantages of this test (MHT) include possible interpretation difficulties and the inability to distinguish between different classes of carbapenemases (Tsakris *et al.,* 2009; Hirsch and Tam, 2010).

Table.1 Detection of carbapenemase production by modified Hodge test and combined disc test among the clinical isolates of Enterobacteriaceae

Clinical isolates	Combined disc test				Modified Hodge test	
	#KPC	$MBL	KPC+MBL	NEGATIVE	POSITIVE	NEGATIVE
Escherichia coli	04	20	01	03	06	22
Klebsieallae pneumoniae	01	08	08	01	08	10
Citrobacter	02	-	-	-	01	01
Enterobacter	02	-	-	-	02	-
Total	**09**	**28**	**09**	**04**	**17**	**33**

#KPC – Klebsiealla producing carbapenemase
$MBL – Metallo-β-lactamase

Fig.1 Detection of Carbapenemase production by modified Hodge test and combined disc test



In recent CLSI document M100-S22, they have mentioned in that, not all carbapenemase-producing isolates of *Enterobacteriaceae* are MHT positive and MHT-positive results may be encountered in isolates with carbapenem resistance mechanisms other than carbapenemase production (CLSI, 2011). However the MHT may not be the ideal phenotypic confirmatory test for all types of carbapenemase producers and false positives have been reported (Carvalhaes, 2011).

In the present study three combined-discs test employing meropenem alone and with PBA, EDTA or both PBA and EDTA were tested for the differentiation of KPCs and MBLs among the carbapenem resistant Enterobacteriaceae species. The present study gave additional information that the

combined-disc test using a boronic acid and EDTA compounds as inhibitors and meropenem as the antibiotic substrate can be successfully used for the identification of KPC and MBL enzymes, among the family *Enterobacteriaceae* such as *E. coli, Klebsiella, Citrobacter* and *Enterobacter species*. The current assay has also proposed an additional combined-disc based on the simultaneous use of both inhibitors, PBA and EDTA, in order to detect the co-production of both MBLs and KPCs. The simultaneous using of both inhibitors seems to restrict the activity of both carbapenemases against meropenem, allowing the detection of isolates that co-produce these enzymes in almost all cases. The present study has clearly shown that in the clinical laboratory the combined use of two inhibitors can clearly detect and differentiate carbapenemase production.

Carbapenem resistant *Enterobacteriaceae* (CRE) have been considered as one of the greatest threats to the global health care in this century. We conclude that modified Hodge test is not a useful method for differentiation of carbapenemase production and combined disc test for MBL and KPC production is accurate, cost-effective, easy to perform and also useful for clinical laboratories to promptly report any isolate with carbapenemase activity to the hospital infection control team, to allow the implementation of appropriate contact isolation precautions.

References

Ambler, R.P., *et al.* 1991. A standard numbering scheme for the class A B-lactamases. *Biochem. J.,* 276: 269–272.

Bansal, M., Vyas, N., Sharma, B., *et al.* 2013. Differentiation of carbapenemase producing Enterobacteriaceae by triple disc test. *Indian J. Basic Appl. Med. Res.,* 3: 314–320.

Baraniak, J., Fiett, M., Herda, E., *et al.* 2013. Species distribution, clonal structure and carbapenemase types of carbapenemase-producing Entero bacteriaceae in European and Israeli hospitals comparative study. www.congrex.ch/.../2013.ECCMID13.

Bratu, S., Landman, D., Haag, R., *et al.* 2005. Rapid spread of carbapenem-resistant *Klebsiella pneumoniae* in New York city: a new threat to our antibiotic armamentarium. *Arch. Intern. Med.,* 165: 1430–5.

Cai, J.C., Zhou, H.W., Zhang, R., *et al.* 2008. Emergence of *Serratia marcens, Klebsiella pneumoniae* and *Escherichia coli* isolates possessing the plasmid-mediated carbapenem-hydrolyzing β-lactamase KPC-2 in intensive care units of a Chinese hospital. *Antimicrob. Agents Chemother.,* 52: 2014–8.

Carvalhaes, C.G. 2010. Cloverleaf test (modified Hodge test) for detecting carbapenemase production in Klebsiella pneumoniae: be aware of false positive results. *J. Antimicrob. Chemother.,* 65: 249–251.

Clinical and Laboratory Standards Institute, 2011. Performance standards for antimicrobial susceptibility testing; 20th informational supplement. CLSI document M100-S21. CLSI, Wyne, PA.

Datta, P., Gupta, V., Garg, S., *et al.* 2012. Phenotypic method for differentiation of carbapenemases in Enterobacteriaceae: study from north India. *Indian J. Pathol. Microbiol.,* 55: 357–60.

Deshpande, P., Rodrigues, C., Shetty, A., *et al.* 2010. New Delhi Metallo-beta-lactamase (NDM-1) in Enterobacteriaceae: Treatment options

with carbapenems compromised. *J. Assoc. Physicians India,* 58: 147–9.

Franklin, C., Liolios, L., Plegy, A.Y. 2006. Phenotypic detection of carbapenem-susceptible metallo-β-lactamase-producing gram-negative bacilli in the clinical laboratory. *J. Clin. Microbiol.,* 44: 3139–44.

Galani, I., Rekastsina, P.D., Hatzaki, D. *et al.* 2008. Evalution of different laboratory tests for the detection of metallo- β-lactamase production in Enterobacteriaceae. *J. Antimicrob. Chemother.,* 61: 548–553.

Giakkoupi, P., Pappa, O., Polemis, M., *et al.* 2009. Emerging *Klebsiella pneumoniae* isolates co-producing KPC-2 and VIM-1 carbapenemases. *Antimicrob. Agents Chemother.,* 53: 4048–50.

Hirsch, E.B., Tam, V.H. 2010. Detection and treatment options for *Klebsiella pneumonia* carbapenemases (KPCs): an emerging cause of multidrug-resistant infection. *J. Antimicrob. Chemother.,* 48: 1323–1332.

Kitchel, B., Rasheed, J.K., Patel, J.B., *et al.* 2009. Molecular epidemiology of KPC-producing *Klebsiella pneumoniae* in the United States: clonal expansion of MLST sequence type 258. *Antimicrob. Agents Chemother.,* 53: 3365–70.

Nordman, P., Cuzon, G., Naas, T. 2009. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infect. Dis.,* 9: 228–36.

Nordman, P., Nass, T., Poirel, L. 2011. Global spread of carbapenemase-producing Enterobacteriaceae. *Emerg. Infect. Dis.,* 17: 1791–1798.

Pasteran, F., Mendez, T., Guerriero, L., *et al.* 2009. Sensitive screening tests for suspected class A carbapenemase production in species of Enterobacteriaceae. *J. Clin. Microbiol.,* 47: 1631–9.

Pasteran, F., Mendez, T., Rapoport, M., *et al.* 2010. Controlling false-positive results obtained with the Hodge and Masuda assays for detection of class A carbapenemase in species of Enterobacteriaceae by incorporating boronic acid. *J. Clin. Microbiol.,* 48: 1323–1332.

Paterson, D.L. 2006. Resistance in gram-negative bacteria: Enterobacteriaceae. *Am. J. Infect. Control,* 34: S64–S73.

Pournara, S., Protonotoarious, E., Voulgari, E., *et al.* 2009. Clonal spread of KPC-2 carbapenemase-producing *Klebsiella pneumoniae* strains in Greece. *J. Antimicrob. Chemother.,* 64: 348–52.

Queenan, A.M., Bush, K. 2007. Carbapenemases: the versatile beta-lactamases. *Clin. Microbiol. Rev.,* 20: 440–58.

Schwaber, M.J., Carmeli, Y. 2008. Carbapenem-resistant Entero bacteriaceae: a potential threat. *J. Am. Med. Assoc.,* 300: 2911–2913.

Tsakris, A., Kristo, I., Poulou, A., *et al.* 2009. Evalution of boronic acid disk tests for differentiating KPC-possessing *Klebsiella pneumoniae* isolates in the clinical laboratory. *J. Clin. Microbiol.,* 47: 362–7.

Tsakris, A., poulou, A., Pournaras, S., Voulgari, E., *et al.* 2010. A simple phenotypic method for the differentiation of metallo-β-lactamases and class A KPC carbapenemases in *Enterobacteriaceae* clinical isolates. *J. Antimicrob. Chemother.,* 65: 1664–71.

Walsh, T.R., Toleman, M.A., Poirel, L., Nordman, P. 2005. Metallo-β-lactamases: the quiet before strom? *Clin. Microbiol. Rev.,* 18: 306–325.