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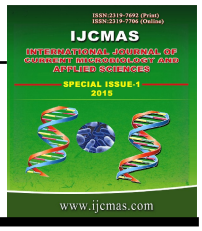

Original Research Article

Experience with a Commercial Assay for Detecting Different Carbapenemases in Enterobacteriaceae

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A B S T R A C T

Metallo-β-lactamases and serine carbapenemases in Enterobacteriaceae are rapidly threatening the utility of carbapenems. This has led to the need for specific diagnostic tests to detect these enzymes with high sensitivity and to discriminate between them. Reliable detection of carbapenemases is necessary to implement contact precautions and for outbreak detection. Our aim was to detect Class A *Klebsiella pneumoniae* carbapenemase (KPC) and similar serine carbapenemases, Class B metallo-beta-lactamases (MBL), and AmpC-type beta-lactamases in carbapenem-resistant Enterobacteriaceae isolates in our teaching hospital, using the proprietary KPC + MBL Detection Kit from Rosco Diagnostica, Denmark. Fifty meropenem-resistant strains isolated at Himalayan Institute of Medical Sciences, Dehradun between November 2013 and June 2014, were analysed with the Rosco KPC + MBL Kit comprising of four disks: meropenem 10 µg (to confirm resistance), meropenem + phenylboronic acid (inhibitor of KPC-type carbapenemases and AmpC-type beta-lactamases), meropenem + dipicolinic acid (inhibitor of MBLs), and meropenem + cloxacillin (inhibitor of AmpC-type beta-lactamases). The dominant species were *Klebsiella pneumoniae* with 22 (44%), *Escherichia coli* with 10 (20%) and *Enterobacter cloacae* with seven (14%) isolates. The remaining isolates included *Enterobacter aerogenes, Citrobacter freundii, Citrobacter koseri, Serratia marcescens, Proteus vulgaris and Proteus mirabilis*. Thirty-five (70%) isolates produced MBL, while two (4%) produced both KPC and MBL carbapenemases. The remaining isolates (26%) produced neither KPC-type serine carbapenemases nor metallo-beta-lactamases. Around a quarter of carbapenem-resistant Enterobacteriaceae isolates tested in our hospital did not produce Class A or B carbapenemases or AmpC-type beta-lactamases and are presumed to have been carbapenem resistant with the help of Class D (OXA-type) carbapenemases, efflux pumps, porin loss or a combination of any of these factors.

Introduction

In the never ending war against bacteria, carbapenem resistance in both community-acquired and nosocomial infections, is one of our biggest challenges. Carbapenems are

active against both Gram-positive and Gram-negative bacteria, including anaerobes, with the exception of intracellular bacteria such as the Chlamydiae (Pitout and Laupland, 2008). Carbapenems have generally been considered to be resistant to older beta-lactamases but increasing resistance to this class of drugs is fast changing that.

Carbapenem-resistant Enterobacteriaceae (CRE) have been reported worldwide, and most strains have become resistant by acquiring carbapenemase genes (Queenan and Bush, 2007). These organisms have become a great concern because of the frequency with which they cause infections, the associated high mortality, and their potential to transmit carbapenem resistance via mobile genetic elements (Gupta *et al.*, 2011). Apart from the expression of carbapenemases, resistance may be related to a decrease in outer-membrane permeability, over-expression of older β-lactamases with poor carbapenemase activity, or to expression of efflux pumps (Bush and Jacoby, 2010). Various carbapenemases have been reported in *Enterobacteriaceae*, including *Klebsiella pneumoniae* carbapenemase (KPC; Ambler class A); Verona integron–encoded metallo-β-lactamase (VIM), imipenemase (IMP), New Delhi metallo-β-lactamase (NDM) (all Ambler class B); and oxacillinase-48 (OXA-48; Ambler class D) (Nordmann *et al.*, 2011). In addition, carbapenemase producers are usually associated with other non–β-lactam resistance determinants, which give rise to multidrug- and pan drug-resistant isolates (Walsh and Tolemon, 2012).

Among the carbapenem resistance mechanisms, carbapenemase production is currently the most frequently encountered. While state-of-the-art techniques such as spectrometry and molecular assays are being developed, their availability and feasibility in the routine laboratory setting is questionable (Lee *et al.*, 2013). In this context, the phenotypic tests have the advantage of being more practicable even in a simple laboratory setting. Different tests have been proposed to detect carbapenemases, using either phenotypic or genotypic techniques. So the present study was carried out to detect Class A *Klebsiella pneumoniae* carbapenemase (KPC) and similar serine carbapenemases, Class B metallo-beta-lactamases (MBL), and AmpC-type beta-lactamases in carbapenem-resistant Enterobacteriaceae isolates in a teaching hospital, using the proprietary KPC + MBL Detection Kit from Rosco Diagnostica, Denmark.

Materials and Methods

The study includes all clinical specimens referred for bacteriological culture from outdoor (OPD) as well as indoor (IPD) patients of all age groups and both sexes from different wards of our hospital. The specimens comprised of urine, blood, sputum, pus, CSF, body fluids, swabs, endotracheal aspirate, tracheal tube aspirate, etc. Enterobacteriaceae isolates were identified by standard biochemical methods.

A total of 50 consecutive meropenem resistant clinical Enterobacteriaceae isolates received in the Department of Microbiology, Himalayan Institute of Medical Sciences, Jolly Grant Dehradun, India, from November 2013 to June 2014 were considered for this analysis. Meropenem resistance was determined using disk diffusion method as per the Clinical and Laboratory Standards Institute (CLSI, 2013) guidelines. These resistant isolates were further evaluated for detection of the KPC and MBL carbapenemases using the Rosco Diagnostica KPC+MBL detection kit.

An inoculum of the test strain was adjusted to a turbidity equivalent to 0.5 McFarland standard and grown on Mueller-Hinton agar plates as lawns. Four disks, namely meropenem (MRP), meropenem + boronic acid (MRPBO), meropenem + dipicolinic acid (MRPDP) and meropenem + cloxacillin (MRPCX) from Rosco Diagnostica kit were placed on each plate. The plates were then incubated at 37°C for 24 hours and the results were interpreted as follows:

Result and Discussion

The 50 meropenem resistant isolates represented different bacterial populations The dominant species were *Klebsiella pneumoniae* with 22 (44%), *Escherichia coli* with 10 (20%) and *Enterobacter cloacae* with seven (14%) isolates. The remaining isolates included *Enterobacter aerogenes*, *Citrobacter freundii*, *Citrobacter koseri*, *Serratia marcescens*, *Proteus vulgaris* and *Proteus mirabilis*.

Of the 50 Enterobacteriaceae isolates tested by Rosco Diagnostica KPC+MBL kit, 35 strains were found to be MBL producers (Figure 1) i.e. the growth inhibitory zone diameter around the Meropenem disc with dipicolinic acid had increased to >=5mm as compared to the growth inhibitory zone diameter seen around the disc containing Meropenem alone. 2 isolates were found to coproduce both MBL and KPC, (Figure 2) as the growth inhibitory zone diameters seen around the Meropenem disc with boronic acid and meropenem with dipicolinic acid had increased to >=5mm as compared to the growth inhibitory zone diameter seen around the disc containing Meropenem alone. The rest of the 13 isolates were negative for both MBL and KPC productions, (Figure 3) as none of the discs showed any inhibitory activity.

Several phenotypic confirmation tests have been described for the detection of carbapenemase-producing *Enterobacteriaceae.* These include bioassays that detect the ability of these enzymes to hydrolyze the carbapenems (e.g., modified Hodge test [MHT]) and inhibitor-based methods using metal chelators for MBLs (e.g., MBL E-test), boronic acid for KPCs (Nordmann *et al.*, 2012), and the commercial systems such as the Mast discs ID inhibitor combination disks and the Rosco Diagnostica Neo-Sensitabs KPC and MBL confirmation kit**.**

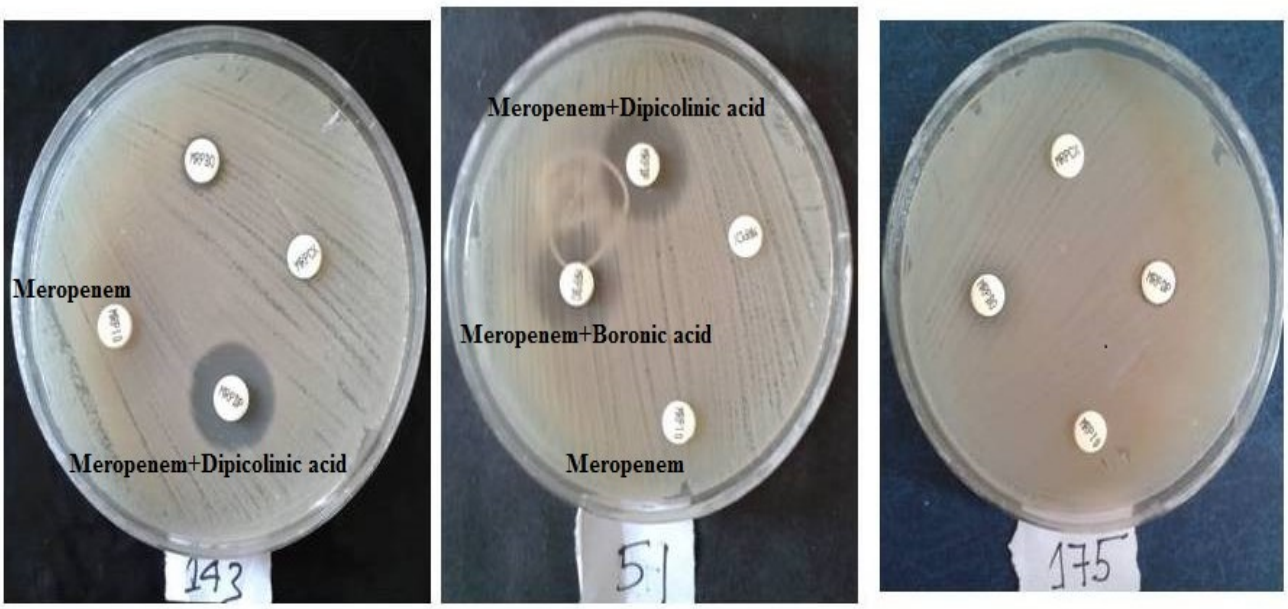
In our study we used Rosco Diagnostica KPC and MBL kit for the detection of different carbapenemases in Enterobacteriaceae. This inhibitor based assay was found to be useful in detecting carbapenemases in Enterobacteriaceae, with MBL being the most predominant mechanism of resistance. Similar findings have been reported in another study, wherein almost 100 per cent sensitivity and specificity have been reported on the use of inhibitor assay for detection of MBL and KPC (Giske *et al.*, 2011). In our study, 2 isolates showed the production of both KPC and MBL. Similar findings were also reported in the study done by Miriagou *et al.* (2013). Inhibitor based assays for detection of MBL and KPC in formats such as epsilometer test, double disc approximation test and combination disc test are currently used as phenotypic confirmatory tests. Among these formats, the combination disc test is reported to perform superior, easier to carry out and interpret (Behera *et al.*, 2008). The Rosco kit employs the combination principle using meropenem and three inhibitors for three types of carbapenemases. Boronic acid is a potent inhibitor of KPC and other types of serine carbapenemases except OXA.

Table.1 Interpretative criteria for Rosco KPC + MBL Detection Kit

Difference between Inhibition zone diameter around various discs			
MRPBO-MRP	MRPDP-MRP	MRPCX-MRP	Putative Carbapenem Resistant Mechanism
≥ 4 mm	< 5 mm	≥ 5 mm	Amp C Production
≥ 4 mm	< 5 mm	< 5 mm	KPC
< 5 mm	≥ 5 mm	< 5 mm	MBL
Key to abbreviations: **KPC**- Klebsiella pneumoniae carbapenemases, **MBL**- Metallo-β-lactamases, **AmpC**- AmpC – type β- lactamases **MRP**- Meropenem, **MRPBO**- Meropenem + Phenylboronic acid **MRPDP**- Meropenem + Dipicolinic acid, **MRPCX**- Meropenem + Cloxacillin			

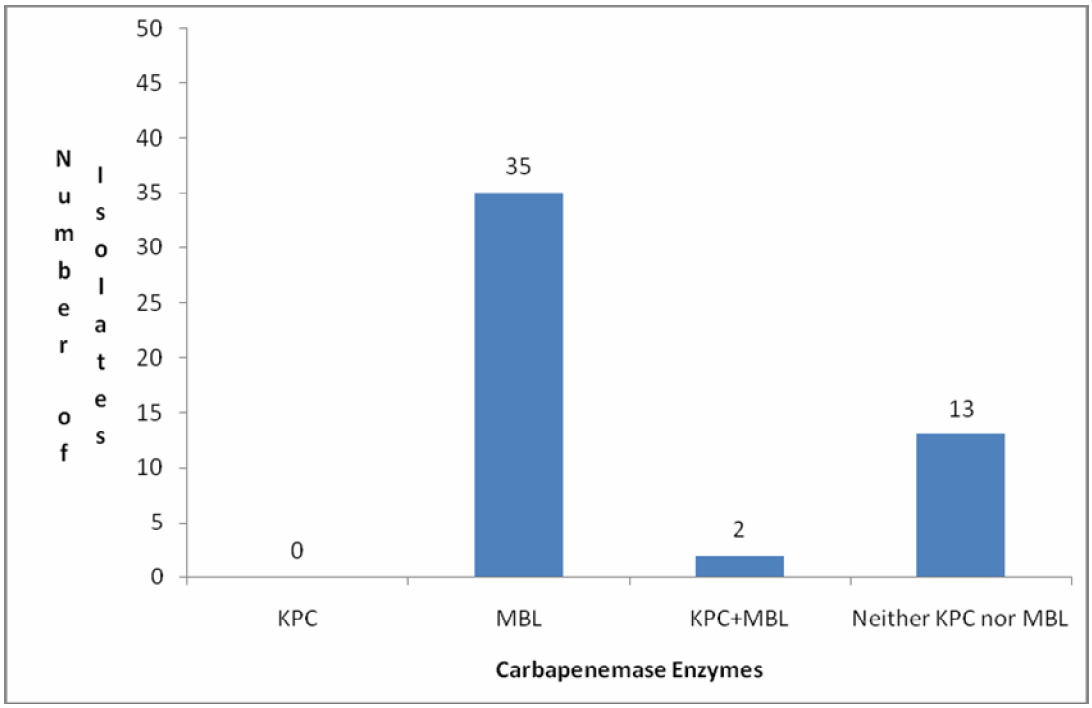
The following strains were used for quality control:
Klebsiella pneumoniae ATCC BAA-1705, KPC Positive
Klebsiella pneumoniae ATCC BAA-2146, MBL Positive
Klebsiella pneumoniae ATCC 700603, Negative control

Figure 1 Figure 2 Figure 3



MBL Positive MBL+KPC Positive Neither KPC nor MBL

Figure.4 Graph showing number of isolates with different carbapenemases



Studies have reported a very high sensitivity and specificity of this compound in detecting KPC (Nicola *et al.*, 2012). EDTA and dipicolinate are potent chelators of divalent metal cations and are hence strong inhibitors of the zinc-dependent MBL. The AmpC-β-lactamase capable of hydrolysing carbapenems is rarely produced by Gram‑negative bacteria including the Enterobacteriaceae (Dahyot *et al.*, 2013). These carbapenemases are inhibited by both boronic acid and cloxacillin (Mirelis *et al.*, 2006). The OXA types of carbapenemases are occasionally found in NFGNB and very rarely in the Enterobacteriaceae (Niranjan *et al.*, 2013). Till date there are no compounds capable of inhibiting this class of carbapenemases. However, high level resistance to temocillin is an exclusive feature of OXA carbapenemases, not seen in other types of carbapenemases. Based on this property, the manufacturers have added a fifth tablet containing temocillin to the existing pack. This new pack is hence capable of identifying all types of carbapenemases at once. (Van Dijk *et al.*, 2013). The clinical laboratory acts as an early warning system, alerting the medical community to new resistance mechanisms present in clinically important bacteria. We believe that the presence of carbapenemases among *Enterobacteriaceae* is an infection control emergency and that the detection of these bacteria in clinical laboratories is a critical step required for appropriate management of patients and infection prevention and control efforts. Clinical microbiology laboratories should be able to rapidly detect these enzymes among members of the *Enterobacteriaceae*, especially when these enzymes are first introduced into the local bacterial population. We recommend using such molecular tests for the optimal detection of these isolates and feel that initially it is important to know what type of carbapenemase is present. Unfortunately, these tests are expensive and often are

available only in large referral or research laboratories.

Our study showed the MBL as the major carbapenemase responsible for the carbapenem resistance in Enterobacteriaceae. The results of the present study correlate well with other recent studies, which suggest the Rosco kit as a reliable test in the detection of carbapenemases. The Rosco kit also has an added advantage that it can identify the class of carbapenemase produced and is also capable of differentiating the co-production of two different classes of carbapenemases by the same isolate. The use of synergic assay allows discriminating between different types of carbapenemases and can be suggested for routine diagnostic application because of its low cost, reliability, and very good discriminatory potential among different resistance mechanisms.

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