Prevalence of $bla_{NDM-1}$ Producing Blood Isolates of *Escherichia Coli*, *Klebsiella* species and *Enterobacter* Species in a Tertiary Care Centre in South India

Maanasa M. Bhaskar, Radha Anand and Harish B N.

Department of Microbiology, Jawaharlal Institute of Post graduate Medical Education and Research centre, Puducherry, India.

E- mail for Correspondence: drbnharish@yahoo.com

ABSTRACT

Carbapenems like meropenem and imipenem are being increasingly used for the treatment of multidrug resistant pathogens especially those producing extended spectrum beta lactamase and AmpC beta lactamase. Since the first usage of antibiotics, the burden of resistance has progressively increased over the past several years. Carbapenemases are the enzymes which are capable of hydrolyzing carbapenems and are a heterogeneous mixture of beta lactamases belonging to Ambler molecular class A (Penicillinases), class B (Metallo enzymes) and class D (oxacillinases). Carbapenem-resistant Enterobacteriaceae (CRE) due to carbapenemase production is being increasingly seen in clinical practice and has been reported worldwide over the past few years. This jeopardizes the effective use of carbapenems and have led to the development of ‘superbugs’. Blood stream infections caused by these CRE are important cause of morbidity and mortality. In the present study, we investigated the prevalence of $bla_{NDM-1}$ producing blood isolates of *Escherichia coli*, *Klebsiella* species and *Enterobacter* species. All the 93 blood isolates of *Escherichia coli*, *Klebsiella* species and *Enterobacter* species collected from October 2010 to March 2012 were either resistant to or intermediate to meropenem by disc diffusion method. Minimum inhibitory concentration determination of meropenem for all these isolates showed varied results. The results of phenotypic and genotypic tests for the presence of carbapenemases showed a prevalence of 70.6% of $bla_{NDM-1}$ among the study isolates. 67% of $bla_{NDM-1}$ was found in *Klebsiella* species, 75% in *Escherichia coli* and 76% in *Enterobacter* species. The indiscriminate and inappropriate use of antibiotics has led to the development of these multi drug resistant pathogens. As there are no antibiotics currently available for the treatment of these organisms, judicial use of antibiotics should be practiced and strict antibiotic policy and hospital infection surveillance should be implemented for halting further spread of these organisms.

Key words: CLSI- clinical laboratory standard institute, CRE- carbapenem resistant Enterobacteriaceae, MIC- Minimum Inhibitory Concentration, MHT- Modified Hodge Test, MBL – Metallo beta lactamase, BMD- Broth microdilution

INTRODUCTION

Blood stream infections are an important cause of morbidity and mortality despite the availability of potent antibiotics and advanced supportive care. Beta lactam antibiotics have been the main stay of treatment for a number of gram positive and gram negative infections. Most active among the beta lactam group of antibiotics are the carbapenems. Members of
this group have a definite role in the empirical and definitive therapy of serious and multidrug resistant Gram negative bacilli (Extended spectrum beta-lactamase (ESBL) and AmpC beta-lactamase producing) especially those belonging to the members of the Enterobacteriaceae family (Harish BN et al., 2007; Mouloudi E et al., 2010)

Resistance to carbapenems started emerging within a decade of its introduction in 1980s and is of increasing concern now (Deshpande LM et al., 2004). There are three main mechanisms by which an organism can confer resistance to carbapenems viz: carbapenem hydrolyzing enzymes, presence of efflux pumps and decreased expression of outer membrane proteins. Out of these, the main mechanism of resistance is by production of carbapenem hydrolyzing enzymes-carbapenemases (Oelschlaeger P et al., 2010). These newer antimicrobial resistant bacteria, carbapenem-resistant Enterobacteriaceae (CRE) mainly Escherichia coli, Klebsiella pneumoniae and Enterobacter species are emerging as a major problem from the health care epidemiology stand point. Antimicrobial resistance in gram negative bacilli is of increasing concern because of the lack of newer antibiotics to treat these infections. Therefore, investigating the mechanism underlying the resistance in these multidrug resistant pathogens has an impact on treatment measures and thereby the outcome (Charan J et al., 2012)

MATERIALS AND METHODS

Bacterial isolates

Present study was a descriptive study conducted in a 1650 bedded tertiary care centre in South India to investigate the prevalence of carbapenemase producing isolates. All consecutive non-duplicate isolates of Escherichia coli, Klebsiella species and Enterobacter species, resistant/intermediate to meropenem by routine antimicrobial susceptibility testing, from blood culture were collected over a period of 18 months (from October 2010 to April 2012) and characterized phenotypically and genotypically for the presence of carbapenem hydrolyzing enzymes.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of all the isolates was performed on Mueller-Hinton agar plates by standard Kirby bauer disc diffusion method as per the standard procedure given in CLSI January 2009 M02-A10 document. The isolates were tested to the following panel of antibiotics including the beta-lactam and the non-beta lactam group of antibiotics. Meropenem 30 μg (Hi-media, Mumbai), In-house prepared discs of ciprofloxacin (5 μg), ceftriaxone (30 μg), ceftazidime (30 μg), amikacin((30 μg) and gentamicin(10 μg). ATCC E.coli 25922 was used as the quality control strain in each series.

Determination of Minimum Inhibitory Concentration (MIC)

MIC of a particular strain, resistant/intermediate to meropenem by routine disc diffusion method, was determined by using meropenem trihydrate pure powder (Orchid Pharmaceuticals, Chennai) by broth microdilution and by E-strip method (bioMe`rieux, SA, France). Doubling dilutions of meropenem ranging from 0.25 μg/mL to 128μg/mL were tested by broth micro dilution method. Results were interpreted according to the CLSI Jan 2011 interpretive MIC break points for carbapenems for members of the Enterobacteriaceae family.

Detection of carbapenem resistance mechanisms

A significantly elevated MIC (or a decreased zone of inhibition in disc diffusion testing) to meropenem was taken as the first indication for further confirmation of that particular strain for the presence of carbapenemase. Once a screen criterion such as elevated MIC of meropenem or imipenem is found, the presence of carbapenemase is further detected by using a number of phenotypic methods like MHT and Double Disc Synergy test (DDST).

Modified Hodge test (MHT)

All the carbapenem resistant or intermediate isolates were checked for the presence of carbapenemases using MHT, also known as the clover leaf test as described by Lee et al 2003. Presence of indentation indicates a positive test and
the isolate is a carbapenemase producing strain. No growth of the ATCC E.coli 25922 along the organism growth streak indicates a negative test and the isolate is not a carbapenemase producer.

Metallo-beta-lactamase detection (MBL)
Several phenotypic methods are available for the detection of MBL production. Inhibition of MBL using metal chelators like EDTA or 2-mercapto propionic acid is the most commonly used one. All the carbapenem resistant isolates were tested using imipenem – I-EDTA E-strips for the presence of MBL production.

A lawn culture of 0.5 Mc.Farland suspension of the test isolate was made. E-strip containing imipenem and Imipenem-EDTA (bioMe’rieux, SA, France) was placed and the plates were incubated at 37°C for 16-24 hours. After incubation, the plates were read the value was taken where the ellipse intersects the scale on the strip. When the ratio of the value obtained for the Imipenem: Imipenem + EDTA is more than 8, it is indicative of metallo beta lactamase production. ATCC Ps.aeruginosa 27853 was used as a negative control and a known in-house positive Ps.aeruginosa was used as a positive control.

Molecular detection of carbapenemase genes
Polymerase chain reaction was performed for the detection of blaNDM-1. The primer sequence and the cycling conditions used were according to Mulvey et al 2011.

RESULTS
Blood culture was done for a total of 17,205 patients (8795 paediatric patients and 8409 adult patients) during the study period of 18 months from October 2010 to April 2012 which yielded 93 isolates (5.4%) which were resistant/intermediate to meropenem [K.spp (n=59), E.coli (n=8), Enterobacter spp (n=26)]. All the isolates were from In-patients. These inpatient isolates were from various Intensive care units (ICUs) or from various wards (41 and 52 out of 93 isolates were from various ICUs and from various wards respectively).

All the isolates were resistant to the beta-lactam antibiotics tested and few were susceptible to gentamicin, amikacin and ciprofloxacin as shown in Figure 1. MIC of meropenem for 93 study isolates was determined by BMD and by E-strip
Figure 2. MIC determinations using meropenem E strip.

Figure 3. MBL detection using imipenem-imipenem-EDTA E- strip

method as shown in Figure 2. MIC of ≤1μg/ml was considered susceptible, 2μg/ml as intermediate and ≥4 μg/ml as resistant. MIC of meropenem for all the study isolates showed varied results as shown in table 1. E-test values agreed with the BMD values for most of the isolates except that the value tended to be higher than those of BMD.

Detection of various carbapenem resistant mechanisms

MHT & Imipenem-imipenem EDTA synergy test

In order to differentiate carbapenemase producers from non-carbapenemase producers, MHT & Imipenem- imipenem – EDTA disc synergy test was done for all the 93 meropenem resistant isolates as shown in Figure 3. Presence of clover leaf type of indentation in MHT was considered to be positive for carbapenemase production as shown in Figure 4. Of the 93 isolates tested, 55 (59.1%) showed clover leaf type indentation and were positive by MHT. 64 (68.8%) of the isolates were positive by Imipinem-I EDTA disc synergy test (which includes the MHT positive isolates also) and 28 (30.1%) isolates were negative by both the tests.

PCR for \textit{bla} \textsubscript{NDM-1} gene

The PCR for carbapenemase gene was done and the gel documentation of simplex PCR for \textit{bla} NDM-1 is shown in Figure 5. The results of the PCR showed that among the 93 isolates tested, 66(70.6%) isolates were \textit{bla} \textsubscript{NDM-1}
1 Positive control- bla<sub>NDM-1</sub> positive Klebsiella pneumoniae strain. 2,3,4 – Test strains.

**Figure 4.** Modified Hodge test using ertapenem disc.

Lane 1- Molecular ladder  
Lane 2- Positive control- Klebsiella pneumoniae bla<sub>NDM-1</sub>.  
Lane 3- Negative control- Double sterile distilled water.  
Lane 4- Blank  
Lane 6 to 8 – Test isolates positive for bla<sub>NDM-1</sub>  
Lane 9-10- Test isolates negative for bla<sub>NDM-1</sub>.

**Figure 5.** Gel documentation of Simplex PCR for bla<sub>NDM-1</sub> genes

producers. Out of 66 bla<sub>NDM-1</sub> isolates 40 (67%) were Klebsiella spp, 6 (75%) from E.coli and 20(76%) from Enterobacter spp. The distribution of bla<sub>NDM-1</sub> among the various carbapenemase producers are depicted in Table 2.

**DISCUSSION**

In the present study, all the isolates were obtained from inpatients (100% from various wards) which indicate that these carbapenem resistant isolates are predominantly nosocomial pathogens. But one must also be aware of the fact that these multidrug pathogens can also be community acquired as CREs have also been isolated from the common water sources (Walsh TR et al., 2011).

All the isolates collected were either resistant or intermediate to meropenem by disc diffusion and further confirmed by doing MIC for meropenem by BMD which was considered as the gold standard and also by E-strip method (Anderson
Table 1. MIC and disc diffusion results of meropenem

<table>
<thead>
<tr>
<th>MIC VALUE FOR MEROPENEM (μg/ml)</th>
<th>TOTAL NO OF ISOLATES</th>
<th>MEROPENEM DISC DIFFUSION RESULTS (No of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Klebsiella species</td>
<td>E.coli</td>
</tr>
<tr>
<td></td>
<td>(n=59)</td>
<td>(n=8)</td>
</tr>
<tr>
<td>1 (μg/ml)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2 (μg/ml)</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>4(μg/ml)</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>8(μg/ml)</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>16(μg/ml)</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>32(μg/ml)</td>
<td>12</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2. The distribution of the \(bla_{NDM-1}\) carbapenemase genes among Klebsiella species, E.coli and Enterobacter spp.

<table>
<thead>
<tr>
<th>Carbapenemase gene(s)</th>
<th>Klebsiella spp (n=59)</th>
<th>Escherichia coli (n=8)</th>
<th>Enterobacter spp (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(bla_{NDM-1})</td>
<td>40 (67%)</td>
<td>6(75%)</td>
<td>20 (76%)</td>
</tr>
</tbody>
</table>

KF et al., 2007; Bulik CC et al., 2010; Mochon AB et al., 2011). Most of the isolates were resistant to the routinely used first line antimicrobial agents. This observation is seen in almost all the studies done on CRE isolates. The frequent co-resistance to other classes of antibiotics seen in CRE isolates is mainly because of the simultaneous presence of other resistance determinants often carried on integrons (Carmeli et al., 2010; Deshmukh D et al., 2011). Analysis of the MIC results showed that the carbapenem MICs varied substantially among these multi drug resistant pathogens. The values range between 2-32μg/ml in carbapenemase producing isolates and between 1-16μg/ml in non-carbapenemase producing isolates.

In the present study, MHT was positive in 59% (55 out of 93 meropenem resistant) of the isolates. This indicates that carbapenemase mediated mechanism of resistance is more frequent than the non-carbapenemase mediated mechanism of resistance. Among the CRE study isolates, \(bla_{NDM-1}\) was detected in 71% (66 out of 93) by PCR. Three isolates had MIC value of 1 μg/ml and 18 isolates had MIC value of 2 μg/ml. 61% of these isolates carried \(bla_{NDM-1}\) gene. This finding is in agreement with other reports which suggest the increasing prevalence of NDM-1 in Indian subcontinent. Deshpande et al., reported a similar finding from Mumbai in which majority of the NDM producing isolates were \textit{Klebsiella pneumoniae} and \textit{E.coli}. Even though NDM-1 was initially identified in a Swedish patient treated in India, study done by Kumarasamy et al., clearly shows that \(bla_{NDM-1}\) has now been reported from almost every continent except South America and Antarctica. The same study shows that \(bla_{NDM-1}\) also coexisted with aminoglycoside resistance genes like OXA-23 and armA.

Until recently, the MBLs have been associated mainly with the non-fermentative bacteria like \textit{Pseudomonas aeruginosa}. It did not draw much attention because of the intrinsic resistance to a variety of antibiotics showed by these non-fermenters. It is a well known fact that the antimicrobial resistance among the commonly isolated organisms like the members of the Enterobacteraceae family is a matter of public health importance globally. Studies on carbapenemase producing \textit{E.coli}, \textit{K.pneumoniae} were limited when compared to those on non-fermenters. However, with the discovery of NDM-1 from India, the scenario has changed and a number of studies were published based on this new MBL enzyme. Studies also have detected the presence of \(bla\) NDM gene among the bacteria isolated from sewage samples and public tap waters from New Delhi, India (Walsh et al., 2011). The indiscriminate use of antibiotics as empirical therapy to treat the multi drug resistant pathogens may be responsible for the emergence of these carbapenemase producing isolates in the hospital settings. CRE isolates often exhibit an extensively drug resistant phenotype, resistant to most of the currently available antibiotics rendering them ineffective and the clinical data on the antimicrobial agents available for the treatment of these isolates remain sparse.
CONCLUSION

Bacteria that are carbapenem resistant mainly due to carbapenemase production are being increasingly seen in clinical practice (Pillai et al., 2011). This jeopardizes the effective use of carbapenems and have led to the development of "Super bugs". The emergence of these carbapenemase producers especially CRE reflects the indiscriminate and irrational use of antibiotics because such strains are undoubtedly an outcome of antibiotic pressure (Nordman et al., 2012). Thus judicial use of antibiotics should be practiced and less severe infections should be treated with non-carbapenem drugs and carbapenems should be reserved for the treatment of severe infections. Active surveillance and hospital infection control policies should be implemented not only in the acute health-care settings but also in the non-acute care facilities along with strict antimicrobial policies to prevent the spread of these multidrug resistant strains (Yang Q et al., 2010).

Limitations of the study

The prevalence of CRE in our hospital setting was not calculated since the study included only blood isolates and isolates from other samples were not included. Another important limitation of the present study was that a correlation between the In-vitro and In-vivo results could not be made since the clinical outcome of these patients from whom the meropenem resistant isolates were isolated were not collected.

REFERENCES


Carbapenem resistance in Enterobacteriaceae: here is the storm! Trends in molecular medicine. May 2012 Vol 18, No.5.


