Simple screening tests for detection of carbapenemases in clinical isolates of nonfermentative Gram-negative bacteria

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Received April 10, 2008

Background & objectives: The production of carbapenemases is an important mechanism responsible for the carbapenem resistance. A simple and inexpensive testing method for screening of carbapenemase producers is essential. A prospective study was undertaken to detect metallo-β-lactamases (MBLs) and AmpC β-lactamases in nonfermentative Gram negative bacteria and to evaluate the various methods for detection of carbapenemases and MBLs.

Methods: A total of 100 Acinetobacter spp. (78 A. baumannii and 22 A. lwofii) and 140 Pseudomonas spp. (103 P. aeruginosa and 37 other Pseudomonas spp.) were screened for meropenem resistance by Kirby-Bauer disc diffusion method. Modified Hodge test, EDTA disk synergy (EDS) test and AmpC disk test were used for the detection of carbapenemases, MBLs and AmpC β-lactamases, respectively.

Results: Forty six (59.0%) A. baumannii, 7 (31.8%) A. lwofii, 32 (31.1%) P. aeruginosa and 7 (18.9%) Pseudomonas spp. were resistant to meropenem. Among the 32 meropenem resistant P. aeruginosa, 15 (46.9%) were AmpC β-lactamase producers, 16 (50.0%) MBL producers by EDS test, but only 9 (28.1%) found positive for carbapenemases by modified Hodge test. Among the 46 meropenem resistant A. baumannii, 31 (67.4%) were AmpC β-lactamase producers, 3 (6.5%) MBL producers, but only 1 (14.3%) was positive for carbapenemases by modified Hodge test. One P. aeruginosa was positive for carbapenemase by modified Hodge test, but was negative for MBL and AmpC β-lactamase.

Interpretation & conclusions: MBL production is an important mechanism of carbapenem resistance among Pseudomonas species but not among Acinetobacter species. EDS is more sensitive for detection of MBLs than modified Hodge test. Both EDTA-meropenem and EDTA-ceftazidime combination must be used to detect all the MBL producers. Carbapenemases other than MBL may also be responsible for carbapenem resistance. AmpC β-lactamase is also a contributory factor for carbapenem resistance among the isolates in the hospital.

Key words AmpC β - lactamases - AmpC disk test - EDTA disk synergy test - metallo-β-lactamases - modified Hodge test - nonfermentative bacteria

One of the most important mechanisms of microbial resistance to β-lactam antibiotics (penicillins, cephalosporins, monobactams, and carbapenems) is hydrolysis by β-lactamases. Genes coding for β-lactamase enzymes mutate continuously in response to the heavy pressure of antibiotic use leading to development of newer β-lactamases with a broad spectrum of activity. Among the β-lactamases,
the carbapenemases especially transferrable metallo-
β-lactamases (MBLs) are the most feared because of
their ability to hydrolyze virtually all drugs in that
class, including the carbapenems. There are several
mechanisms for carbapenem resistance such as the
lack of drug penetration due to mutation in porins,
loss of certain outer membrane proteins and efflux
mechanisms.

In addition to their resistance to all β-lactams,
the MBL producing strains are frequently resistant
to aminoglycosides and fluoroquinolones. However,
these usually remain susceptible to polymyxins. Unlike carbapenem resistance due to several other
mechanisms, the resistance to MBL and other
carbapenemase production has a potential for rapid
dissemination, as it is often plasmid mediated.
Consequently, the rapid detection of carbapenemase
production is necessary to initiate effective infection
control measures to prevent their dissemination.

**Material & Methods**

A prospective study was conducted over a period
of nine months (June 2007 to February 2008) at the
Department of Microbiology, Jawaharlal Institute
of Postgraduate Medical Education and Research
(JIPMER), Puducherry. A total of 100 *Acinetobacter*
species and 140 *Pseudomonas* species were included
in this study. These organisms were isolated from
specimens like sputum, tracheal aspirate, pus, urine,
blood, pleural fluid and ascitic fluid of patients admitted
to different wards, which were sent to the microbiology
laboratory for routine culture identification and
sensitivity testing.

The *Acinetobacter* species and *Pseudomonas*
species were identified based on standard
bacteriological techniques. All these isolates were
screened for meropenem resistance by Kirby-Bauer
disk diffusion method according to CLSI guidelines.

**Modified Hodge test:** The meropenem resistant strains
were subjected to modified Hodge test for detection
of carbapenemases. An overnight culture suspension
of *Escherichia coli* ATCC 25922 adjusted to 0.5
McFarland standard was inoculated using a sterile
cotton swab on the surface of a Mueller-Hinton agar
(MHA) (HI-MEDIA, Mumbai, India). After drying,
10 µg meropenem disk (HI-MEDIA, Mumbai, India)
was placed at the center of the plate and the test
strain was streaked from the edge of the disk to the
periphery of the plate in four different directions. The
plate was incubated overnight at 37°C. The presence
of a ‘cloverleaf shaped’ zone of inhibition due to
carbapenemase production by the test strain was
considered as positive (Fig. 1).

**EDTA disk synergy (EDS) test:** EDTA disk synergy
(EDS) test was done with simultaneous testing of two
different β-lactams (meropenem and ceftazidime), for

![Fig. 1. Modified Hodge test. Positive strain shows a ‘cloverleaf shaped’ zone of inhibition due to carbapenemase production, while the negative strain shows an undistorted zone of inhibition.](image-url)
detection of metallo-β-lactamases in the meropenem resistant isolates.

A 0.5 M EDTA solution was prepared by dissolving 186.1 g of disodium EDTA.ZH2O (REACHEM, Chennai, India) in 1,000 ml of distilled water. The pH was adjusted to 8.0 by using NaOH (HI-MEDIA, Mumbai, India) and was sterilized by autoclaving.

An overnight liquid culture of the test isolate was adjusted to a turbidity of 0.5 McFarland standard and spread on the surface of a MHA plate. A 10 µg meropenem disk or 30 µg ceftazidime disk (HI-MEDIA, Mumbai, India) was placed on the agar. A blank disk (6 mm in diameter, Whatmann filter paper no. 1) was moistened with sterile saline and inoculated with a few colonies of the test strain. The inoculated disk was then placed beside the cefoxitin disk almost touching it. The plate was incubated overnight at 37°C. A flattening or indentation of the cefoxitin inhibition zone in the vicinity of the disk with test strain was interpreted as positive for the production of AmpC β-lactamase. An undistorted zone was considered as negative.

**Results**

Of the 100 clinical isolates of Acinetobacter species, 78 were A. baumannii, while 22 were A. Iwoffii. Among the 140 Pseudomonas isolates screened, 103 were P. aeruginosa, while the remaining 37 were other Pseudomonas spp.

Forty six (59.0%) A. baumannii, 7 (31.8%) A. Iwoffii, 32 (31.1%) P. aeruginosa and 7 (18.9%) Pseudomonas spp. were found resistant to meropenem.

Among the 32 meropenem resistant P. aeruginosa, 15 (46.9%) were AmpC β-lactamase producers, 16 (50.0%) were MBL producers by EDTA disk synergy test, but only 9 (28.1%) were positive for carbapenemases by modified Hodge test (Table). Two isolates were positive for both MBL and AmpC β-lactamase. One was positive for carbapenemase by modified Hodge test, but was negative for MBL and AmpC β-lactamase by EDTA disk synergy test and AmpC disk test respectively. Of the 16 MBL producers, 8 were detected by simultaneously testing with both meropenem and ceftazidime in EDS, 6 were detected only using EDTA-ceftazidime combination and 2 were positive by EDTA-meropenem combination alone.

Of the 7 meropenem resistant Pseudomonas spp., 4 (57.1%) were AmpC β-lactamase producers, 2 (28.6%) were MBL producers by EDTA disk synergy test, but only 1 (14.3%) was positive for carbapenemases by modified Hodge test (Table). Of the 2 MBL producers, one was detected by simultaneously testing with both meropenem and ceftazidime in EDS, while the other was detected only using EDTA-ceftazidime combination.
Among the 46 meropenem resistant *A. baumannii*, 31 (67.4%) were AmpC β-lactamase producers, 3 (6.5%) were MBL producers, but only 1 (14.3%) was positive for carbapenemases by modified Hodge test (Table). Among the 3 MBL producers, one was detected by simultaneously testing with both meropenem and ceftazidime in EDS and 2 were detected only using EDTA-ceftazidime combination.

Of the 7 meropenem resistant *A. lwoffi*, 4 (57.1%) were AmpC β-lactamase producers, but none were positive for MBL and other carbapenemases (Table).

### Discussion

Carbapenems have a broad spectrum of antibacterial activity, and these are resistant to hydrolysis by most β-lactamasies including extended spectrum β-lactamasies (ESBL) and AmpC β-lactamasies. These are often used as a last resort in infections due to multidrug-resistant Gram-negative bacilli. However there is an alarming increase in reports of carbapenem resistance in *Acinetobacter* species and *P. aeruginosa*. The first metallo-β-lactamase-producing *P. aeruginosa* strain was isolated in Japan in 1988. For many years, these MBL producing isolates were restricted to Japan, but now it has disseminated worldwide. In India, MBL producing *P. aeruginosa* was first reported in 2002.

In our study we report a high prevalence of meropenem resistance among *A. baumannii*. In a prospective study with 150 *Acinetobacter* species conducted at Bangalore, India, 14.0 per cent resistance to carbapenems was reported. Surveillance in Brooklyn, New York, revealed that approximately 2 of every 3 isolates of *Acinetobacter* species were resistant to carbapenem. In an Australian study with ninety *A. baumannii* isolates recovered from blood, 64 per cent resistance to meropenem was noted. Though our result was contradictory to the relatively lesser resistance rates among *Acinetobacter* species documented in India, it was similar to the New York and Australian studies, which also have reported high prevalence of carbapenem resistance among *Acinetobacter* species. High antibiotic pressure due to indiscriminate use of carbapenems in this part of the world could have resulted in the increase in carbapenem resistant *Acinetobacter* spp.

Among the *P. aeruginosa* 31.1 per cent meropenem resistance was documented in this study. A 5 year longitudinal study involving many centers from Latin America has reported that, *P. aeruginosa* resistance to carbapenems has risen to 40.0 per cent. In a study done at a tertiary care hospital in Puducherry (India), with 266 isolates of *P. aeruginosa*, 10.9 per cent resistance to carbapenems was reported. Though the carbapenem resistance among *P. aeruginosa* in our study was lesser than the Latin American surveillance study, it was higher than the resistance documented in 2006 from this part of the world. These findings clearly show a rising trend in the carbapenem resistance among the nonfermenters.

We also found that 50 per cent of the carbapenem resistance among *P. aeruginosa* was attributable to the production of MBLs. This was surprisingly higher than the documented report in Korea, where 11.4 per cent of imipenem-resistant *P. aeruginosa* isolates produced MBLs. But another Indian study has reported MBL production among 75 per cent of the imipenem-resistant *Pseudomonas* species. Among the meropenem resistant *A. baumannii*, 6.5 per cent were MBL producers, which was relatively less compared to 14.2 per cent imipenem-resistance attributable to the production of MBLs reported in Korea. In an Indian study on meropenem resistant *Acinetobacter* species none of the isolates produced MBLs. Consequently in the Indian scenario production of MBL may not play a major role in carbapenem resistance among *Acinetobacter* species.

EDTA disk synergy test detected MBL production in additional 9 *Pseudomonas* species and 2 *Acinetobacter* species, which were missed by the modified Hodge test. Based on these findings, EDTA disk synergy test seems to be a better method for MBL detection then modified Hodge test. Though the reason for the difference in the performance of these two tests is not clear, similar results have been observed in other studies. In the EDS test, EDTA-ceftazidime combination detected additional MBL producers which were not identified by EDTA-meropenem.

### Table: Results of modified Hodge test, EDTA disc synergy test & AmpC disc test

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>No. of meropenem resistant isolates</th>
<th>No. of positives (%)</th>
<th>Modified Hodge test</th>
<th>EDTA disc synergy test</th>
<th>AmpC disc test</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td>32</td>
<td>9 (28.1)</td>
<td>16 (50.0)</td>
<td>15 (46.9)</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas spp.</em></td>
<td>7</td>
<td>1 (14.3)</td>
<td>2 (28.6)</td>
<td>4 (57.1)</td>
<td></td>
</tr>
<tr>
<td><em>A. baumannii</em></td>
<td>46</td>
<td>1 (2.2)</td>
<td>3 (6.5)</td>
<td>31 (67.4)</td>
<td></td>
</tr>
<tr>
<td><em>A. lwoffi</em></td>
<td>7</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (57.1)</td>
<td></td>
</tr>
</tbody>
</table>
combination. The reason for the increased sensitivity of EDTA-ceftazidime combination is the ability of ceftazidime to produce a marked inhibitory effect with EDTA. Therefore, ceftazidime appears to be the better substrate for EDS. Similar results were observed in a study done at the National Institute of Infectious Diseases, Tokyo, Japan.28

Two MBL producing P. aeruginosa were detected only by EDTA-meropenem combination, but not by EDTA-ceftazidime combination. A synergistic zone of inhibition which must have been normally present between EDTA and ceftazidime due to MBL production was not observed with these two isolates. These two MBL producers were also simultaneously producing AmpC β-lactamase. Hence it could be proposed that the synergistic zone of inhibition was masked by the resistance to ceftazidime conferred by the AmpC β-lactamase, which is independent of zinc ions for its action. Based on this study it is clear that both EDTA-meropenem and EDTA-ceftazidime combination must be used simultaneously to detect all the MBL producers, which may otherwise be missed by using either of this combination alone.

One of the meropenem resistant P. aeruginosa was positive for carbapenemase by modified Hodge test, but was negative for MBL and AmpC β-lactamase. This may be because of the production of carbapenemase other than MBL, which is not dependent on zinc ion for its action. Accordingly, apart from MBL, other classes of carbapenemases (class A or D) can also be responsible for meropenem resistance in P. aeruginosa.29

AmpC β-lactamase was produced by 46.9 and 67.4 per cent of the meropenem resistant P. aeruginosa and A. baumannii respectively. Therefore, AmpC β-lactamase could be an important contributory factor for meropenem resistance among the isolates in our hospital similar to other studies.22,30,31 This seems to be more likely with A. baumannii, most of which in our study, despite being meropenem resistant, did not show production of MBL or other carbapenemases.

The detection of MBL and other carbapenemases is of utmost importance in deciding the most appropriate therapeutic regimen for treatment of the carbapenem resistant nonfermenters. In several studies, intravenous colistin combined with rifampin and imipenem was recommended for the treatment of carbapenem-resistant isolates lacking MBLs, whereas the combination of colistin and rifampicin (with or without tigecycline) was recommended for treatment of MBL producing carbapenem-resistant isolates.30,31 Moreover, studies have shown that efflux pumps mainly affect meropenem, while specific β-lactamases (carbapenemases) hydrolyze imipenem more efficiently.30 So, detection of carbapenemases is necessary for administration of appropriate therapy.

The drawbacks in our study are the relatively small sample size and failure to evaluate the clinical usefulness of detection of carbapenemases. Our study was restricted mainly to detection of carbapenemases and the comparison of the efficacy of different techniques for detection of carbapenemases. Further studies are needed to evaluate the clinical usefulness of detection of carbapenemases.

In conclusion, MBL production is an important mechanism of carbapenem resistance among Pseudomonas species, while it may not play a major role in carbapenem resistance among Acinetobacter species. Modified Hodge test may not be a useful screening test for carbapenemases as many MBL producing isolates were not detected by this test. EDS is a more sensitive method for detection of MBLs. Ceftazidime appears to be the better substrate for EDS compared to meropenem, but both meropenem and ceftazidime must be used simultaneously to detect all the MBL producers. Carbapenemases other than MBL may also be responsible for carbapenem resistance in these nonfermenters. AmpC β-lactamase is also a contributory factor for carbapenem resistance among the isolates in the hospital.

References


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