

Comparison of two methods to detect carbapenemase & metallo- β -lactamase production in clinical isolates

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Background & objectives: Bacterial resistance has greatly hampered effective treatment of patients in clinical settings. Non-fermenting Gram-negative bacilli (NFGNB) are common nosocomial pathogens. In this study we attempted to develop a convenient test for early detection of carbapenemase and metallo- β -lactamase (M β L) production in NFGNB. Lack of sufficient reports from India in this area indicated the need for this study.

Methods: A total of 50 imipenem resistant NFGNB were speciated, and their resistance reconfirmed by disk diffusion and minimum inhibitory concentration (MIC) determination by agar dilution. Two different methods namely modified Hodge and EDTA disk synergy tests were evaluated for carbapenemase and metallo- β -lactamase (M β L) production.

Results: Of the 50 imipenem resistant NFGNB, 48 and two respectively fell in the resistant and intermediate range in MIC using agar dilution. Majority of these were *Pseudomonas aeruginosa* (n=28), followed by *Burkholderia cepacia* (n=9). The modified Hodge test could detect 28 strains as carbapenemase and M β L producers, while the EDTA disk synergy test was able to detect an additional 8 strains producing M β L and carbapenemase.

Interpretation & conclusion: *Pseudomonas aeruginosa* was found to be the predominant NFGNB in our hospital setting and EDTA disk synergy could detect more carbapenemase and metallo- β -lactamase producers compared to modified Hodge test.

Key words Disc synergy tests - metallo- β -lactamases - modified Hodge test EDTA - non-fermenting Gram-negative bacilli

The ability to produce β -lactamase enzymes is the major cause of resistance of bacteria to β -lactam antibiotics and has been the subject of extensive microbiological, biochemical and genetic investigations¹. Numerous β -lactamases exist, encoded either by chromosomal genes or transferable genes

located on plasmids or transposons. Based on amino acid and nucleotide sequences studies, four distinct classes of β -lactamase have been defined namely, classes A and C using serine as an active site residue, class B (the metal- β -lactamase) using Zinc and class D enzymes or OXA-enzymes which are also serine based but quite distinct from classes A or C².

The introduction of carbapenem into clinical practice represented a great advance for the treatment of β -lactam resistant bacteria. Due to their broad spectrum of activity and stability to hydrolysis by most β -lactamase, the carbapenems have been the drugs of choice for treatment of infections caused by penicillin- or cephalosporin-resistant Gram-negative bacilli. However, this scenario is changing with the emergence of metallo- β -lactamase producing strains, especially the non fermenting Gram-negative bacilli (NFGNB).

The modified Hodge test uses *Escherichia coli* ATCC 25922 and 10 μ g imipenem disc instead of *Staphylococcus aureus* ATCC 25923 and a 10 μ g penicillin disc, respectively^{3,4}. The double disk synergy test⁴ has been modified using EDTA instead of a β -lactamase inhibitor. These tests detect and differentiate carbapenemase and metallo- β -lactamase production from other β -lactamases⁴.

A simple reliable test to detect carbapenemase and the metallo- β -lactamase production is useful particularly in situations where carbapenem and β -lactams are commonly used in therapeutic regimen. There is not much information available from India in this area. We therefore undertook this study to evaluate the usefulness of these two tests for the detection of carbapenemase and metallo- β -lactamase production in imipenem resistant clinical isolates of NFGNB.

Material & Methods

A total of 50 consecutive NFGNB, resistant to imipenem, isolated from blood, broncho alveolar lavage (BAL) and sputum specimens from patients admitted to different wards in the Christian Medical College, Vellore between December 1999 and December 2001 were included in the study. All 50 NFGNB were characterized to the species level using standard procedures⁵.

Antibiotic susceptibility testing was performed by the Kirby Bauer disc diffusion method⁶. This was further confirmed by determining the minimum inhibitory concentration (MIC) of imipenem for the strains by agar dilution⁷ using imipenem concentrations ranging from 0.062-128 μ g/ml in Mueller-Hinton agar (DIFCO, USA). *E. coli* American Type Culture Collection (ATCC) 25922 and

Pseudomonas aeruginosa ATCC 27853 were used as controls. The organism was considered to be susceptible if MIC was <4 μ g/ml and resistant if MIC was >16 μ g/ml⁸.

Imipenem resistant strains were checked for production of carbapenemase and metallo β -lactamase by the modified Hodge test and EDTA disc synergy test⁴.

Modified Hodge test was carried out on Mueller-Hinton agar. The plate was inoculated using a cotton swab dipped in an overnight culture suspension of *E. coli* ATCC 25922. (Opacity of the tube was adjusted by comparing with a 1:10 dilution of 0.5 McFarland opacity standard⁹). After brief drying, 10 μ g imipenem disc was placed at the center of the plate and test strains were streaked from the edge of the disc to the periphery of the plate in four different directions. After overnight incubation the plates were observed for the presence of a 'cloverleaf shaped' zone of inhibition. The plates with such zones were interpreted as modified Hodge test positive.

For the EDTA-disk diffusion synergy test an overnight broth culture of the test strain, (opacity adjusted to 0.5 McFarland opacity standard⁹) was used to inoculate a plate of Mueller-Hinton agar. After drying, a 10 μ g imipenem disc and a blank filter paper disk (6 mm in diameter, Whatmann filter paper no. 2) were placed 10mm apart from edge to edge, 10 μ l of 0.5 M EDTA (Sigma, USA) solution was then applied to the blank disc, which resulted in approximately 1.5 mg/disc. After overnight incubation, the presence of an enlarged zone of inhibition was interpreted as EDTA synergy positive.

Results & Discussion

Of the 50 NFGNB, 28 (56%) were *Pseudomonas aeruginosa*, followed by 9 isolates of *Burkholderia cepacia*, 5 of *Sphingomonas pacimobilis*, 2 each of *Sphingobacterium spiritworum* and *Burkholderia pseudomallei*, 1 each of *Burkholderia gladioli*, *Chryseobacterium meningosepticum*, *Flavimonas oryzihabitans* and *Bregyella zoohelcum* respectively.

Of the 50 strains resistant to imipenem by disc diffusion, 48 were resistant and two were in the intermediate MIC range. The modified Hodge test detected 28 strains as carbapenemase and metallo- β -

lactamase producers, 16 were *P. aeruginosa*. The EDTA disc synergy test detected the same 28 strains as well as an additional eight strains as carbapenemase and M β L producers; 21 were *P. aeruginosa*. The likely reason for the resistance in the remaining isolates may be mechanisms other than metallo- β -lactamase production such as decreased membrane permeability¹⁰.

In this study the predominant NFGNB showing imipenem resistance were *P. aeruginosa*, with 76 per cent of these coming from BAL and sputum specimens, indicating that these were important sources of spread. These *P. aeruginosa* isolates were also resistant to aminoglycosides like gentamicin, amikacin and the quinolones. The modified Hodge test is a simple method for screening for metallo- β -lactamase production but occasional false positives have been reported in literature¹¹. It has been stated that these can be avoided by adding 10 μ l of 50 mM zinc sulphate (140 μ g/disk) to the imipenem disc or by incorporating a final concentration of 70 μ g/ml into the Mueller-Hinton agar¹¹. However, we have not done this in our study.

The EDTA disc synergy test was able to detect eight more strains not detected by the modified Hodge test.

However, the reason why there is better detection by EDTA disc synergy test as compared to modified Hodge test is unclear.

Arakawa *et al*¹² have reported another modification of double disk synergy test which made use of ceftazidime and 2-mercaptopyruvic acid. These tests were evaluated on ceftazidime resistant isolates and detected IMP-1 whereas the EDTA double disk method detected VIM-2 metallo- β -lactamase.

Unfortunately these enzymes are seen in NFGNB where therapeutic options are severely limited. Therefore, detection of carbapenemase and metallo- β -lactamases is a valuable tool in the diagnostic microbiology laboratory as it contributes towards the optimal treatment of patients and control of the spread of resistance. The metallo- β -lactamase efficiently hydrolyzes all β lactams, *in vitro*, except aztreonam¹³.

The implementation of a simple reliable laboratory method to detect carbapenemase and metallo- β -lactamase production is useful particularly in

situations where carbapenem and other β -lactams are indicated or preferred as therapeutic regimen. NCCLS documents do not yet contain a method for detection of metallo- β -lactamase production in isolates, and hence the methods standardized here could be of use. Based on the findings of our study we conclude that the EDTA disc synergy test is better than modified Hodge test for detection of carbapenemase and metallo- β -lactamases. This method might be useful in clinical laboratories to monitor the emergence of carbapenemase and metallo- β -lactamase enzymes for clinical and surveillance network.

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