



Original Research Article

Comparative study of three phenotypic methods for detection of Metallo- β -lactamases in clinical isolates of *Pseudomonas aeruginosa*

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The acquisition of metallo- β -lactamases (MBL) by *P. aeruginosa* has recently emerged as one of the most worrisome resistance mechanisms, a virtue by which they can hydrolyze all beta-lactam antibiotics including penicillins, cephalosporins and carbapenems, with the exception of aztreonam. This study was undertaken to detect and compare production of metallo- β -lactamases in clinical isolates of *P. aeruginosa* by three different phenotypic methods. This study was conducted over a period of one year (January to December 2012), in the Department of Microbiology, Pt. B.D. Sharma, Post Graduate Institute of Medical Sciences, Rohtak. A total of 100 clinical isolates were included in the study and their antimicrobial susceptibility was determined by Kirby-Bauer disc diffusion method. The screening for MBL detection was done by imipenem-EDTA combined disc test, imipenem-EDTA double disc synergy test and EDTA disc potentiation test using four cephalosporins, in the Imipenem resistant isolates. Imipenem resistance was seen in a total of 65 *P. aeruginosa* isolates. Screening for MBL was positive in 57 isolates by combined disc test, 56 isolates by double disc synergy test and 54 isolates by disc potentiation test. Conclusion: Imipenem-EDTA combined disc test was superior to other methods for MBL detection in clinical Microbiology laboratories.

Introduction

Pseudomonas aeruginosa, a commonly encountered nosocomial pathogen, is a potentially troublesome cause of wound infection, urinary tract infection, and respiratory tract infection, especially in immunocompromised patients, thus, inflicting significant morbidity and mortality, worldwide (Khan, 2008). Emergence of resistance to commonly used antibiotics like beta-lactams has been recognized as a cause of treatment failure (Carmelli, 1999). In *P. aeruginosa*, resistance to carbapenems mediated by production of metallo beta lactamases (MBLs) is being increasingly reported

(Manoharan, 2010). The MBLs are associated with a peculiar property of conferring upon the organism a broad-spectrum resistance profile. The presence of MBLs is usually associated with accompanying resistance to β -lactams, aminoglycosides and fluoroquinolones as well, although remaining sensitive to polymyxins. Therefore, a rapid screening method to detect MBLs in multidrug resistant *P. aeruginosa* isolates, could help in modifying therapy and initiating effective infection control measures to prevent further dissemination.

Presently, no one standardized phenotypic method for MBL detection has been proposed. Despite polymerase chain reaction being a highly accurate and reliable method, its accessibility is often limited to reference laboratories. The present study was thus conducted with an objective to detect the prevalence of MBLs in *P. aeruginosa* isolates by three different phenotypic methods and compare their relative efficacy.

Material and Methods

This study was conducted over a period of one year (January to December 2012), in the Department of Microbiology, Pt. B.D. Sharma, Post Graduate Institute of Medical Sciences, Rohtak, a tertiary level health care providing facility in North India.

A total of 100 isolates of *P. aeruginosa* recovered from clinical specimens like urine, pus, blood, body fluids, sputum, etc were included in the study. The clinical isolates recovered from both outdoor and indoor patients, irrespective of their age and gender, were identified following standard microbiological procedures (Collee, 1996a,b).

Antimicrobial Susceptibility

Antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion method as per CLSI guidelines (CLSI, 2011).

Following antimicrobials discs were tested:- ceftazidime (30µg), cefepime (30µg), ceftriaxone (30µg), ceftizoxime (30µg), cefoxitin (30µg), imipenem (10µg), meropenem (10µg), piperacillin/tazobactam (100/10µg), ticarcillin/clavulanic acid (75/10µg), aztreonam (30µg), gentamicin (10µg), amikacin (30µg), netilmicin (30µg), ciprofloxacin (5µg), polymyxin B (300

units), colistin (10µg). In case of urinary isolates, ofloxacin (5µg) and norfloxacin (10µg) were also included. *P. aeruginosa* ATCC 27853 strain was employed as the control strain (Winn Jr et al, 2006; Sussman, 1998).

An isolate was considered as multi drug resistant (MDR), if resistance was encountered to least three of the following classes of antimicrobial agents, i.e β-lactams, carbapenems, aminoglycosides, and fluoroquinolones; and, extremely drug resistant if found to be resistant to all the four classes of antimicrobial agents mentioned previously.

The protocol for the MBL detection in the *P. aeruginosa* isolates by the three methods used in the study is given as under:

Imipenem (IMP)-EDTA combined disc test

The test organism was inoculated on MHA plate as per CLSI guidelines. Two 10µg imipenem discs were placed on the surface of agar plate and EDTA solution was added to one disc to obtain a desired concentration of 750µg. The inhibition zone of imipenem and imipenem-EDTA disc was compared after 16-18 hours of incubation at 35°C. A positive test was indicated if zone enhancement with EDTA impregnated imipenem discs was ≥ 7 mm than imipenem disc alone (Yong et al, 2002).

Imipenem (IMP)-EDTA double disc synergy test (DDST)

Test organism was inoculated on MHA and a 10µg imipenem disc was placed 20 mm centre to centre from a blank disc containing 10µl of 0.5M EDTA (750µg). Enhancement of the zone of inhibition in the area between imipenem and the EDTA disc in comparison

with the zone of inhibition on the far side of the drug was interpreted as a positive result (Lee et al, 2003).

EDTA disc potentiation using ceftazidime, ceftizoxime, cefepime and cefotaxime

Test organism was inoculated on to the Mueller-Hinton agar plates as per CLSI guidelines and a filter paper blank disc was placed. Following discs [ceftazidime (30µg), ceftizoxime (30µg), cefotaxime (30µg), cefepime (30µg)] were placed 25mm centre to centre from the blank disc. Ten µl of 0.5M EDTA solution was added to the blank disc and the plate was incubated overnight at 35⁰C. Enhancement of the zone of inhibition in the area between the EDTA disc and any one of the four cephalosporin discs in comparison with the zone of inhibition on the far side of the drug was interpreted as a positive result (Arkawa et al, 2000).

Result and Discussion

Production of MBL by *P. aeruginosa* and other gram negative bacteria has tremendous impact on therapeutic regimens, since these organisms also carry other multidrug resistance genes and the only viable treatment options remains the administration of potentially toxic polymyxin B and colistin (Livermore and Woodford, 2000). In the present study also, 74% of the MBL producing *P. aeruginosa* isolates were found to multi drug resistant (MDR) and 60% were extremely drug resistant (XDR) isolates. MBL producing strains are more likely to cause invasive disease and are associated with a higher hospital case-fatality rate. Therefore, it becomes important to have a rapid screening test to detect MBLs in these isolates so that further dissemination can be prevented (Noyal et al, 2009).

Since there is no one test considered as gold

standard for detection of MBL, different studies have reported results using different methods. In the current study, out of 100 isolates of *P. aeruginosa*, imipenem resistance was observed in 65.0% of the isolates. Out of these isolates, 87.7% were MBL producers by imipenem (IMP)-EDTA combined disc test, 86.15% by Imipenem (IMP)-EDTA(DDST) and 83.07% by EDTA disc potentiation test(Table.1). The difference in production rates was however not statistically significant. Similarly other studies have also reported 85.7% and 66.7% detection of MBL by imipenem (IMP)-EDTA combined disc test, whereas MBL detection by Imipenem (IMP)-EDTA (DDST) has been reported to be 64.28%, 50.0% and by EDTA disc potentiation test has been reported to be 0.0%, 11.1% (Behera et al, 2008; Bhalerao et al, 2010). This suggests that imipenem (IMP)-EDTA combined disc test is superior to the other two methods. Silpi et al reported 90.2% MBL production in imipenem resistant *P. aeruginosa* isolates by imipenem (IMP)-EDTA combined disc test, imipenem (IMP)-EDTA double disc synergy test and MBL E-test strip, thus suggesting all the three methods to be equally effective in detecting MBL production (Silpi et al, 2012).

The prevalence of MBL detection by imipenem-EDTA combined disc test has been reported to range from 8.0%-85.7% in various studies (Collee et al, 1996; Noyal et al, 2009; Behera et al, 2008; Livermore, 2002). So, the rate of MBL production was comparatively higher in the current study. This can be explained by the fact that because of increase in ESBL producing isolates, use of carbapenems to treat serious infections in our institute has increased and thus lead to antibiotic selection pressure under the influence of which organisms starts expressing many resistance mechanisms for its survival. This might be responsible for production of MBL enzymes

in *P. aeruginosa* isolates in the present study (Noyal et al, 2009; Agarwal et al, 2008; Navneeth et al, 2002). However, another author reported a higher rate (94.20%) of MBL production in imipenem resistant *P. aeruginosa* isolates by imipenem (IMP)-EDTA combined disc test, which can be due to the fact that study was performed only on

isolates recovered from burn wound samples (Saderi et al, 2010). Absence of MBL production in imipenem resistant isolates may be due to other resistance mechanisms involved such as permeability mutations via loss of porins or upregulation of efflux systems which may be missed by phenotypic tests (Saderi et al, 2010).

Table.1 Comparison of three methods employed for the detection of MBL among imipenem resistant *P. aeruginosa* isolates

Total Number of imipenem resistant <i>P. aeruginosa</i> isolates out of 100	MBL Isolates, detected by imipenem (IMP)-EDTA combined disc test	MBL Isolates, detected by imipenem (IMP) - EDTA double disc synergy test (DDST)	MBL Isolates, detected by EDTA disc potentiation method
65	87.7%	86.15%	83.07%

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