

Phenotypic detection of Metallo- and AmpC β -lactamases producing strains of *P. aeruginosa* in the state of Himachal Pradesh (India)

Naveen Minhas and Prakash Chand Sharma*

Department of Microbiology, School of Biotechnology, Shoolini University of Biotechnology and Management Sciences, Bajhol, Solan-173212, Himachal Pradesh, India

Abstract: *Background:* A prospective study was undertaken to detect metallo- β -lactamases (MBLs) and AmpC β -lactamases in *Pseudomonas aeruginosa* isolates recovered from patients at Indira Gandhi Medical College, Shimla (H.P). *Objective:* The study aimed at determining the prevalence of MBL and AmpC producing *P. aeruginosa* isolates. *Material and Methods:* A total of 180 isolates were preliminarily screened for their susceptibility to carbapenems (imipenem, meropenem, doripenem and ertapenem) and cefoxitin by *in vitro* antibiotic cultural sensitivity. The confirmation of MBL producing isolates was done by combined disc test and E test whereas AmpC production was confirmed by disc antagonism test and E test. *Results and Conclusion:* *P. aeruginosa* isolates (22.22%) were MBL producers on the basis of resistance to imipenem and meropenem while 60% were AmpC β -lactamase producers in preliminary screening test. Of the MBL producers, 50% isolates were confirmed by E test and 45% by combined disc test. However, only 13.33% were positive by both the confirmatory tests. By disc antagonism test, 46.33% isolates were detected as AmpC producers while 6.67% were positive by E test. It is interesting to note that of the 180 isolates examined, 14 (7.78%) were both MBL and AmpC producers. Both MBL and AmpC as mechanisms of resistance were thus, observed among the isolates of *P. aeruginosa* in the state of Himachal Pradesh. Emergence of such strains is of public health concern as such organisms pose therapeutic challenge.

Keywords: Metallo- β -lactamases, AmpC β -lactamases, carbapenems.

Introduction

Pseudomonas aeruginosa is one of the most diverse and ecologically significant bacterium among the *Pseudomonas* species. This organism is of greater significance due to widespread distribution of its strains in nature, their resistance to many antibacterial compounds and the number of virulence factors produced by them [1]. *P. aeruginosa* is an aerobic gram negative rod, usually 1.5-5 μ m in length, 0.5 to 1.0 μ m in width and is motile due to the presence of flagella.

This organism is a leading opportunistic and nosocomial pathogen among immunocompromised individuals such as patients suffering from AIDS, cancer, burn wounds and cystic fibrosis. Aminoglycosides, fluoroquinolones, cephalosporins and carbapenems are commonly used antibiotics for treating *P. aeruginosa* infections. However, a decreased susceptibility rate of *P. aeruginosa* to β -lactams, carbapenems, quinolones and aminoglycosides has been

reported from various countries. Among the carbapenems, imipenem is the better therapeutic option as this drug is considered to be most effective. However, resistance to carbapenems has been on the rise alarmingly in recent years [2].

Resistance of *P. aeruginosa* isolate to multiple drugs is usually the result of combination of different mechanisms in a single isolate. Among such mechanisms, over expression of efflux pump, acquisition of extended spectrum β -lactamases (ESBLs), AmpC β -lactamases and metallo- β -lactamases (MBLs), target site or outer membrane modifications are predominant [3]. Beta-lactamases are hydrolytic enzymes which cleave the beta-lactam ring and act as primary mechanism of conferring bacterial resistance to beta-lactam antibiotics [4]. Ambler classified MBLs and AmpC β lactamases as class B and class C respectively [5].

Genetic control of beta-lactamase production resides either on plasmids or on the chromosome, with the potential to move between bacterial populations. AmpC β -lactamases are the enzymes which are not inhibited by clavulanic acid and usually confer resistance to all beta-lactams, with the exception of methoxy-imino-cephalosporins, such as cefepime and the carbapenems. MBLs can hydrolyze all clinical beta-lactam substrates, with the exception of aztreonam [6]. They require divalent cations of zinc as co-factors for enzymatic activity and are universally inhibited by ethylenediamine tetra acetic acid (EDTA). In India, the prevalence of MBLs ranges from 7.5% to 71 % [3]. Resistance to broad-spectrum beta-lactams, mediated by extended-spectrum beta-lactamases (ESBL), AmpC beta-lactamase, and metallo-beta-lactamase (MBL) enzymes, is an increasing problem worldwide [7].

In the present study, occurrence of different β -lactamases (MBLs and AmpC) producing *P.aeruginosa* isolates recovered from pus, urine, blood, sputum specimens of human patients suffering from various disease conditions at Indira Gandhi Medical College, Shimla, Himachal Pradesh during one year period has been investigated. The susceptibility and resistance patterns of these isolates against different cephalosporins and carbapenems have been determined, so as to select most resistant phenotypes for further molecular characterization. This would be helpful in formulating an effective antibiotic strategy and to plan a proper hospital infection control strategy to prevent further spread of these strains.

Material and Methods

Bacterial isolates and their identification: A total of 200 clinical isolates of *P. aeruginosa* were collected over a period of one year from various clinical samples at the department of Microbiology, Indira Gandhi Medical College, Shimla, Himachal Pradesh. Out of these, 180 isolates were confirmed as *P. aeruginosa* by standard conventional methods in Microbiology laboratory of Shoolini University at Solan. These isolates were maintained on slants of Pseudomonas isolation agar (Himedia, Mumbai) and preserved in 80% glycerol at -80°C . Subculturing was done on regular basis in order to maintain the fresh cultures for the experiment.

P. aeruginosa ATCC 27853 was included as quality control strain.

Screening of isolates for MBL production: The *in vitro* antibiotic cultural sensitivity of *P. aeruginosa* for MBL production was done by the disc diffusion method [8]. The following antibiotics were used in the screening test: imipenem (10 μg), meropenem (10 μg), doripenem (10 μg) and ertapenem (10 μg) (Himedia Mumbai, India). The size of zone of inhibition was read after overnight incubation at 37°C and interpreted according to recommended MBL screening criteria as specified in CLSI protocol, M100-S22 [9].

Confirmatory methods

Imipenem-EDTA combined disc test: MBL production was confirmed by IMP-EDTA combined disc test as described by [10]. Imipenem disc (10 μg) and Imipenem/EDTA disc (10 μg /750 μg) (Himedia) were placed on the dried plate 10mm apart from edge to edge. After 24 hours of incubation at 37°C , the inhibition zones of IMP and IMP/EDTA discs were compared. For MBL producing organisms, discs with IMP/EDTA increased inhibition zones by 8 to 15 mm, while the increase of such zones for MBL negative isolates were 1 to 5 mm according to CLSI guidelines, 2013 [9].

MBL E – test: MRP/MRP+EDTA E -test strips (Himedia) consisted of Meropenem (MRP) (4-256 $\mu\text{g}/\text{ml}$) and MRP (1-64 $\mu\text{g}/\text{ml}$) plus constant level of EDTA. An inoculum (0.5 Mc Farland standards) was prepared from 24 hour old culture of the test strain, inoculated on Muller Hinton Agar plate with the help of sterile cotton swabs. After brief drying, E test strips were applied on MHA plates and incubated for 18-24 hours at 37°C . The MIC end points were read where the inhibition ellipses intersected the strips. A ratio of MICs of the Meropenem (MRP) to MRP+EDTA of ≥ 8 was interpreted as MBL positive [9]. *S. maltophilia* ATCC 13636 and *P. aeruginosa* ATCC 27853 were used as positive and negative control strains respectively in the MBL E- test.

Screening of isolates for AmpC production: *P. aeruginosa* isolates were screened for AmpC

β -lactamases by standard disc diffusion breakpoint for ceftazidime. Isolates with zone diameter less than 18 mm for ceftazidime were considered as probable AmpC producers which were further confirmed by other methods [11].

Confirmatory methods

Disc Antagonism Test: Ceftazidime disc (30 μ g) was placed at a distance of 20mm from ceftazidime disc (30 μ g) on a MHA plate inoculated with test organism as per CLSI guidelines. Isolates showing blunting of ceftazidime zone of inhibition adjacent to ceftazidime disc were screened positive for AmpC β -lactamase production. Also strains showing reduced susceptibility to ceftazidime and ceftazidime disc were considered positive for AmpC β -lactamase production [12].

AmpC E-test: The test strains were inoculated on to MHA. E test strip was coated with mixture of four different antibiotics with or without clavulanic acid and tazobactam on a single strip in a concentration gradient manner. The upper half had ceftazidime, cefotaxime, cefepime and cloxacillin mixture + clavulanic acid and tazobactam (MIX⁺) with highest concentration tapering downwards, whereas lower half is similarly coated with ceftazidime, cefotaxime, cefepime and cloxacillin mixture (MIX) in concentration gradient in reverse direction. The results were interpreted as per recommendations of the manufacturers as follows: if the value of the ratio between MIC for MIX and MIC for MIX⁺ was equal to or more than 8, the isolate was considered AmpC producer.

Results

Confirmation of *P. aeruginosa* isolates: A total of 200 clinical isolates were collected from Indira Gandhi Medical College (IGMC) at Shimla and out of which 180 were confirmed as *P. aeruginosa* and screened for MBL and AmpC production.

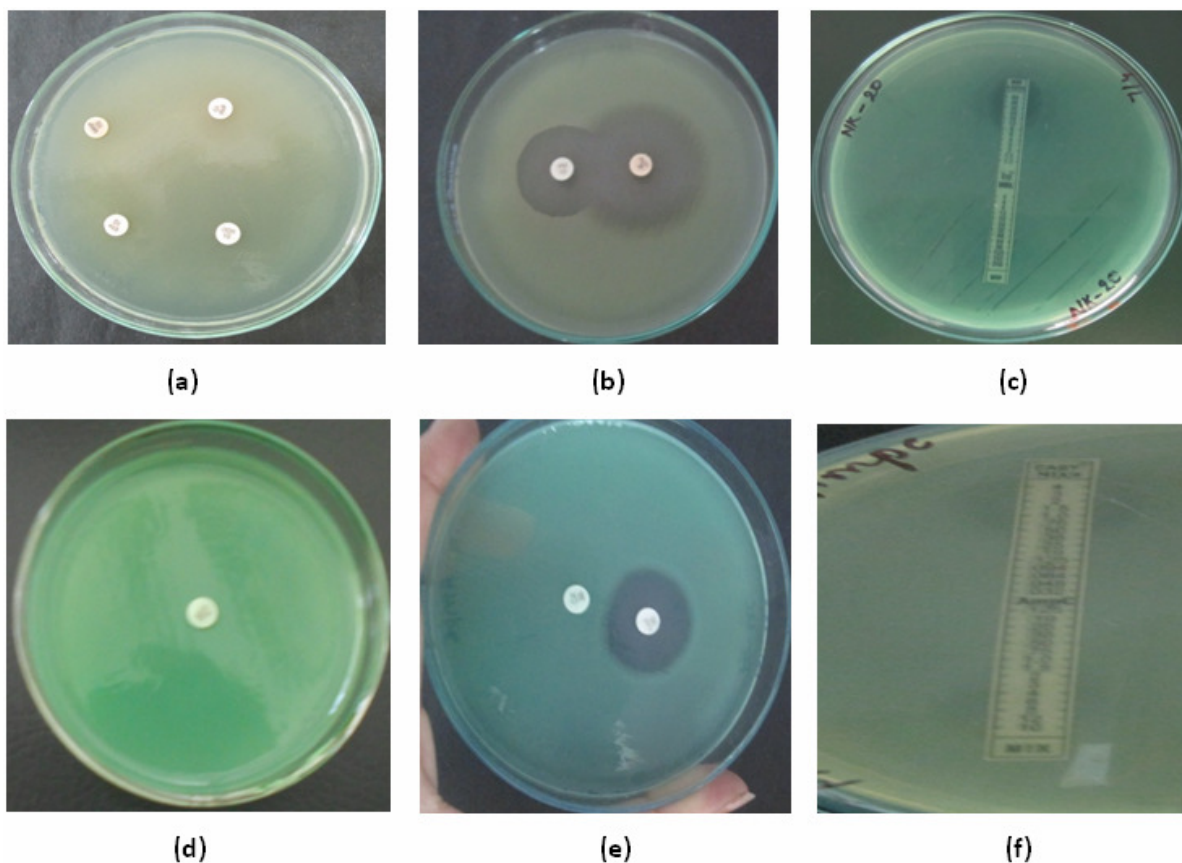
Phenotypic detection of MBL producers and their confirmation: The results of *in vitro* antibiotic cultural sensitivity to different carbapenems are presented in [Table 1]. Majority of isolates (33.89%) were resistant to imipenem followed by ertapenem (23.89%). However, lower proportions of isolates were recorded resistant to doripenem and meropenem and ranged from 6.67% to 14.44% respectively [Table 1, Fig 1(a)].

| Antibiotics | Sensitive | Resistant |
|---------------------------|--------------|--------------------|
| Imipenem | 119 (66.11%) | 45+16*=61 (33.89%) |
| Meropenem | 155 (86.11%) | 14+11*=25 (13.89%) |
| Doripenem | 168 (93.33%) | 10+2*=12 (6.67%) |
| Ertapenem | 137 (76.11%) | 30+13*=43 (23.89%) |
| * Intermediate resistance | | |

A total of 40/180 (22.22%) isolates of *P. aeruginosa* were resistant to one or both carbapenems (imipenem and meropenem) used in the screening test. Of the 40 Imipenem and/or Meropenem resistant isolates 18 (45%) exhibited a zone size enhancement of \geq 7mm in the combined disc test [Table 2, Fig 1(b)]. Majority (55%) isolates were non MBL producers. Out of 30 isolates, 15 (50%) isolates were MBL producers by MBL E-Strip test [Table 2, Fig 1(c)]. Both these tests reflected discordance between the results for most of the isolates, only 4 isolates gave positive results for MBL production by both the tests [Table 2]. *Stenotrophomonas maltophilia* ATCC 13636 and *P. aeruginosa* ATCC 27853 were used as positive and negative quality control strains respectively in these assays.

| No. of isolates resistant to Imipenem and Meropenem (n=180) | Combined disc test (n=40) | E test (n=30) | MBL positive by CDT and E test | Total |
|---|---------------------------|---------------|--------------------------------|-------|
| 40 | 18 | 15 | 4 | 29/40 |

Figure-1: (a) Isolates showing resistance towards carbapenems; (b) and (c) Isolate no. Pa146 is positive for MBL by both phenotypic tests (combined disc test and E - strip test); (d) Isolate showing resistance to ceftazidime disc; Isolate no. Pa85 (e) and (f) is positive for AmpC by both phenotypic tests (disc antagonism test and E - strip test)



| Screening Positive for AmpC production (n=180) | Confirmatory Positive | | AmpC positive isolates by disc antagonism test and E test | Total positive isolates |
|--|------------------------------|---------------|---|-------------------------|
| | Disc antagonism test (n=108) | E test (n=30) | | |
| 108 | 50 | 2 | 0 | 52/108 |

Phenotypic detection of AmpC producers and their confirmation: A total of 108/180 (60%) isolates of *P. aeruginosa* were screened positive for AmpC β- lactamase production by standard disc diffusion breakpoint for ceftazidime [Table 3, Fig 1(d)]. Of the 108 ceftazidime resistant isolates, 50 (46.3%) showed blunting of ceftazidime zone of inhibition adjacent to ceftazidime disc in the disc antagonism test [Table 3, Fig 1(e)]. Majority (53.7%) isolates were non AmpC producers. Out of 30 isolates, 2 (6.67%) isolates were positive for AmpC production by AmpC E-Strip test [Table 3, Fig 1(f)].

*Phenotypic detection of both MBL and AmpC producing *P. aeruginosa* isolates:* A total of 14 isolates were found producers of both MBL and AmpC [Table 4].

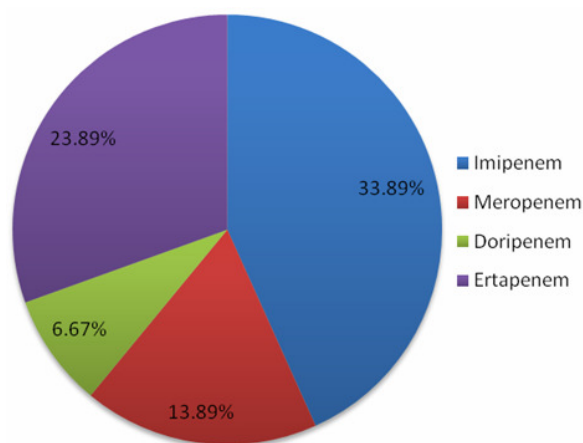
| MBL producer | AmpC producer | Both (MBL + AmpC) |
|--------------|---------------|-------------------|
| 29/40 | 52/108 | 14 |

Discussion

P. aeruginosa is currently one of the most frequently isolated nosocomial pathogen and the infections due to this organism are often difficult to treat due to resistance to various antibiotics. The production of Metallo β -lactamase (MBL) is one among various resistance mechanisms found in *P. aeruginosa*. Prevalence of MBL producing clinical isolates of *Pseudomonas* species have been continuously reported globally with increasing frequency over the past few years and the strains producing these enzymes have been responsible for prolonged nosocomial outbreaks followed by serious infections. As MBL producing *Pseudomonas* species poses a therapeutic problem, it is better to understand the mechanism and spread of such strains which will aid in proper diagnosis and infection control management [1].

In India, prevalence rate of MBLs ranging from 7 – 65% has been reported from various parts of the country: 12% MBL producing *P. aeruginosa* have been reported from Bangalore [13], (8.2%) from Kolkata [14] and (14%) from Chennai [15], 20.8% from Mumbai [16], 8.05% from Nagpur [17], 11.4% from Wardha (MH) [18]. In the present study we observed 72.50% (29/40) MBL positive *P. aeruginosa* isolates which is even higher as compared to earlier study (46.55%) in this region [19].

Fig-2: Pie chart showing percentage of resistance towards carbapenems



Carbapenems are the only reliable active antibiotics against many multi resistant gram negative pathogens particularly those with extended spectrum betalactamases (ESBLs) and

AmpC enzymes. Wide spread use of carbapenems also increases the problem of MBL production. In our study, 33.89% isolates were resistant to imipenem, 23.89% isolates were resistant to ertapenem followed by meropenem (14.44%) and doripenem (6.67%) [Fig.2], while lower rates 9%, 9.8%, 9.9% of imipenem resistance have been reported by other workers respectively [20-22] while higher rates 32%, 71.4%, 59%, 69% and 55% of resistance were reported respectively by others [23-27].

A simple screening test using combined disc diffusion test has been very useful to screen MBL positive isolates. We screened MBL producers among imipenem and meropenem resistant isolates by two methods, the combined disc method and E - strip test for MBL screening and found discordance in the results obtained by these methods as only 4 (10%) of the carbapenem resistant isolates were MBL producers by both the tests [Table 2]. Out of 40 positively screened isolates, 18 (45%) were confirmed through combined disc test and 15 (50%) out of 30 positively screened isolates were confirmed positive through E - strip test for MBL production. 28.89% and 22.22% positive isolates for MBL production by combined disc test and MBL E – strip test respectively were reported by Patwardhan *et al*, 2013 [28] which is lower in incidence as compared to our study.

Organisms over expressing AmpC beta-lactamases are a major clinical concern because these are usually resistant to all beta lactam drugs except for cefepime, ceftipime and carbapenems. Failure to detect AmpC β -lactamase producing strains has contributed to their uncontrolled spread and therapeutic failures. Hence their appearance in a hospital setting should be indentified quickly so that appropriate antibiotic usage and containment measures can be implemented.

Higher incidence of AmpC among *P. aeruginosa* (55.5%) has been reported from South India [29] and even higher rate to the tune of 59.4% has been reported from Uttar Pradesh [4]. In the present study, we observed 48.14% AmpC positive *P. aeruginosa* isolates among cefoxitin resistant isolates whereas low

rate (20%) has been reported from Aligarh [30], 17.3% from Kolkata [12], 22% from a tertiary care hospital in northern India [31] and 16.4% from Pondicherry [32]. In our study, 50/108 (46.29 %) isolates were positive by disc antagonism test whereas 2/30 (6.66%) isolates were positive by E - test strip for AmpC production. According to our data disc antagonism test is more sensitive as compared to E test. Sensitivity of E test can be developed by increasing the drug concentration on the strip. As mentioned in Table 4, out of 81 isolates analyzed for beta lactamases (29MBL + 52AmpC), only 14 produced both of these enzymes.

Conclusion

The present study highlights the prevalence of AmpC β -lactamase and MBL producing *P.*

aeruginosa isolates which may cause outbreaks in population and causes increased morbidity and mortality in the affected patients. Also therapeutic options may be limited due to the high proportion of resistant bacteria. There is necessity to undertake continued surveillance of the resistant organisms and their underlying mechanisms so as to reduce or control further spread of the infections due to *P. aeruginosa*.

Acknowledgements

Authors are thankful to Dr. Vineeta Sharma, Dept. of Microbiology, IGMCI, Shimla for providing the isolates and Prof. P. K. Khosla, Vice Chancellor, Shoolini University of Biotechnology and Management Sciences, Bajhol, Solan (H.P.) for providing necessary facilities to carry out the research work.

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*All correspondences to: Dr. Prakash C. Sharma, Professor, Department of Microbiology, School of Biotechnology, Shoolini University of Biotechnology and Management Sciences, Bajhol, Solan-173212, Himachal Pradesh, India. E-mail: dr.sharmapc@gmail.com