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ORIGINAL ARTICLE

METALLO-ß-LACTAMASE PRODUCTION IN IMIPENEM RESISTANT STRAINS OF PSEUDOMONAS AERUGINOSA

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ABSTRACT

Background: Nosocomial infections are major health issues in developing as well as developed countries. The objective of this study was to determine the frequency of *MBL* production in *Pseudomnas aeruginosa* that causes resistance to Imipenem and other ß-lactam antibiotics.

Materials and Methods: A sample of 52 Imipenem resistant *Pseudomnas aeruginosa* colonizing or infecting the hospitalized patients were collected in Department of Pathology, Post Graduate Medical Institute, Lady Reading Hospital, Peshawar from June 2014 till May 2016. The organisms were identified by routine laboratory tests including biochemical methods and API NE System (Biomeriux) and the sensitivity pattern of commonly used antibiotics was established for each of these isolates using the disc diffusion method. Imipenem resistant strains were tested for *MBL* production by Imipenem-EDTA disc diffusion method.

Results: The frequency of *MBL* activity was positive in 39 (75%) cases of *Pseudomnas aeruginosa* which encodes resistance to Imipenem and other ß-lactam antibiotics except monobactam. The sensitivity pattern of these antibiotics was as follows: piperacillin/ tazobactam 30.8%, amikacin and polymyxin B each 17.9%, tobramycin 12.8%, cefoperazone/ sulbactam and ceftazidime each 5.1%, ciprofloxacin, moxifloxacin, colistin sulphate, tetracycline, azithromycin and aztreonam each 2.6% and co-trimoxzole, gentamicin & rifampin each 0%.

Conclusions: *MBL* production in *P. aeruginosa confers* a challenge for clinicians to treat such resistant infections with conventional antibiotics. Therefore testing each Imipenem resistant *Pseudomnas aeruginosa* for *MBL* production must be taken in routine consideration.

KEY WORDS: Nosocomial infections; Pseudomnas aeruginosa; ß-lactam resistant; Imipenem; Imipenem resistant strains; Antibiotics; Monobactams; Carbapenems; EDTA, Amikacin.

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INTRODUCTION

Pseudomods are gram negative straight or slightly curved rods. They are motile by means of one or more polar flagella. They are non-sporing and non acid-fast, strict aerobes, but some grow anaerobically in the presence of nitrate.[1]

Widespread use of antibiotics in hospitalized patients may over time increase the prevalence of infection with resistant organisms. *MBL* is a novel type of enzyme first reported from Japan in 1991 and presently from almost all parts of the world.[2] This enzyme has the potential to hydrolyze all betalactam antibiotics including carbapenems with the sole exception of monobactam. Moreover, it is resistant to action of serine beta lactamase inhibitors like clavulanic acid and sulbactam. In fact these inhibitors are used as substrates by *MBL*.[3,4]

Over the last decade there have been several studies summarizing the level of *MBLs* in the bacterial community.[5] In the past 5-6 years many new types of *MBLs* have been studied. These include IMP, VIM, SPM, GIM, SIM and the recently discovered AIM.[6] These enzymes are rapidly spreading amongst gram negative organisms. It is not known whether this resistance of *Pseudomonas aeruginosa* to Imipenem is due to *MBL* production or other mechanisms (such as lack of antibiotics penetration due to outer membrane protein mutation and active efflux pumps). This differentiation is important because *MBLs* spreads rapidly

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in *Pseudomonas aeruginosa* due to their integron borne location. *MBL* positive isolates may show in vitro susceptibility to some of the ß-lactam antibiotics but their in vivo activity (except for monobactam) will be reported as resistant.[3,5]

Therefore, *MBL* mediated Imipenem resistance is more of challenge for infection control than other forms of Imipenem resistance in *Pseudomonas aeruginosa*. Detection of *MBLs* leads to prompt measures to check their dissemination and has valuable impact on infection control.

The objective of this study was to determine the frequency of *MBL* production in Pseudomnas aeruginosa that causes resistance to Imipenem and other ß-lactam antibiotics.

MATERIALS AND METHODS

The cross-sectional study was carried out in the Department of Pathology, Lady Reading Hospital, Peshawar, Pakistan from June 2014 till May 2016. A sample of 52 non-duplicate nosocomial isolates of Imipenem resistant *P. aeruginosa* was selected.

Inclusion criterion was all isolates of nosocomial origin that have been collected after 48 hours of admission to the hospital. Repeat isolates of the same species of all specimens (pus, sputum, urine and blood) of the same patients were excluded.

The organisms were identified by routine laboratory tests including biochemical methods and API-NE (analytical prophylactic index-non enterrobacteriacea) (bioMérieux, Inc. Durham, NC, USA) and the sensitivity pattern of commonly used antibiotics was established for each of these isolates using the disc diffusion method. Imipenem resistant strains were tested for *MBL* production by Imipenem-EDTA disc diffusion method.

All relevant isolates of *P. aeruginosa* resistant to Imipenem were stored in Tryptic Soya Broth (TSB) (Oxoid Limited, Hampshire, UK), containing 50% (v/v) glycerol. TSB was mixed with glycerol to make 50% TSB-glycerol broth. It was then autoclaved at 121 0C for 15 minutes at 15lbs/sq in pressure. The broth was then filled in autoclaved Eppendorf tubes under strict asepsis. About 1.5-2 ml of broth was filled in each tube. The tubes were then incubated at 4-8 0C for 24 hours. 3-4 colonies of each bacterial isolate were picked with sterilized wire loop and inoculated into the 50% TSB-glycerol broth, and then incubated overnight. Stock cultures were then frozen at 70 0C in a freezer located in an area of the laboratory to which there was limited access.

A loop full of the stored 50% TSB-glycerol broth with the pure inoculum was sub-cultured onto Cysteine Lactose Electrolyte Deficient (CLED) agar plates. These were then incubated at 35 0C overnight to recover the bacteria as a pure growth.

Standard laboratory procedures for identification of the organisms included colony morphology, Gram staining, growth at 420C, pigment production and several biochemical tests. The latter included oxidase, triple sugar iron (TSI), oxidation fermentation test, catalase test and motility test. Where required, API 20 NE identification systems of bioMérieux were used.

Test organisms were inoculated on two plates of Mueller-Hinton agar as recommended by the CLSI. A 0.5 EDTA solution was prepared by dissolving 186.1 g of disodium EDTA 2H2O (Sigma-Aldrich, Inc., St. Louis, Missouri, USA) in 1,000 ml of distilled water and adjusting it to pH 8.0 by using NaOH. The mixture was sterilized by autoclaving. Two 10-μg-Imipenem discs (Oxoid) were placed on the plate, and appropriate amount of EDTA solution was added to one of them to obtain the desired concentration. The inhibition zones of the Imipenem and Imipenem-EDTA discs were compared after 16 to 18 hours incubation in air at 350C.

We used two criteria; Criteria 1 was that *P. aeruginosa*, all of the *MBL* positive isolates are well separated from *MBL* negative isolates by the criterion of a $\geq$ 7 mm increase of inhibition zone with the disc (Imipenem-EDTA) to which 750 μg of EDTA was added as compared with Imipenem disc alone.

Criteria 2 was that the inhibition zones with Imipenem EDTA discs are $<$ 14 mm for the *MBL* negative isolates, while they are $\geq$ 17 mm for the *MBL* positive isolates.

In order to remove the confusion we modified the criteria 2 to criteria 2A using STOKES comparative analysis, which was defined as to subtract the difference in zones of the EDTA- Imipenem from EDTA and that difference of up to 3 mm distance between the test and control in non-determinable isolates were added to *MBL* negatives.

Sensitivity pattern of Imipenem non susceptible Pseudomnas aeruginosa to alternative antibiotics was determined and percentages calculated. Comparative analysis of *MBL* positive strains of Pseudomnas aeruginosa isolates from different body specimens and various hospital units were carried out using chi-square test. P. values less than 0.05 was considered significant.

An EDTA stock solution is stable, but addition of the solution at each performance of the test is time consuming. To determine the stability of Imipenem discs containing 750 μg of EDTA, the dried discs were stored at 4 or –20 0C without desiccant to simulate the most unfavorable laboratory conditions for 12 to 16 weeks. The inhibition zone for the CLSI control strains (*P. aeruginosa* ATCC 27853) was within the acceptable range for at least 24 weeks.

MBL activity (present/ absent) and zone of inhibition in mm were two research variables. Both being categorical data, were analyzed by count and percentages manually.

RESULTS

Out of a sample of 52 isolates, on criteria 1, based on an increase in the zone of inhibition by ≥ 7 mm with the Imipenem EDTA disc compared with the Imipenem disc alone. By this criteria, 50 (96.2%) isolates were positive for MBL while 2 (3.8%) were negative for *MBL* activity. (Table 1)

Table 1: *MBL* activity of Imipenem resistant *P. aeruginosa* isolates on Criteria 1

MBL Activity	Count	Percentage
Positive	50	96.2
Negative	2	3.8
Total	52	100

By this criteria 2, 44 (84.61%) isolates were positive for *MBL* activity, 6 (11.54%) were negative and 2 (3.85%) were non-determinable. (Table 2)

Table 2: *MBL* activity of Imipenem resistant *P. aeruginosa* isolates on Criteria 2

MBL Activity	Count	Percentage
Positive	44	84.61
Negative	6	11.54
Non-determinable	2	03.85
Total	52	100

EDTA has its own bactericidal activity as was confirmed by applying same quantity of EDTA (as in Imipenem-EDTA discs) to blank discs. We observed zones of inhibition ranging from 8 mm to 20 mm (mean 12.87 mm). (Table 3a)

Table 3a: Zones of inhibition for EDTA (Alone) against IMP-resistant *P. aeruginosa* isolates

Zone size	Count	Percentage
08-12 mm	26	50.00
13-16 mm	22	42.31
17-20 mm	04	07.69
Total	52	100

We observed zones of inhibition with Imipenem-EDTA ranging from 12 mm to 25 mm (mean 19.23mm) (Table 3b).

Table 3b: Zones of inhibition for IMP-EDTA against IMP-resistant *P. aeruginosa* isolates

Zone size	Count	Percentage
12-17 mm	09	17.31
18-21 mm	33	63.46
22-25 mm	10	19.23
Total	52	100

It is therefore, conceivable that EDTA activity alone have contributed to some of the isolates being reported false positive. We modified the above mentioned criteria to avoid confounding false positive results.

For Criteria 2 we placed all those isolates which had a zone of inhibition with EDTA alone of ≥16 mm in the non-determinable category. Keeping in view this modified criteria Criteria 2A, we determined 39 (75%) of the isolates to be positive for *MBL* activity, 6 (11.5%) negative and 7 (13.5%) in non-determinable category. (Table 4)

Table 4: *MBL* activity of Imipenem resistant *P. aeruginosa* isolates on Criteria 2A

MBL Activity	Count	Percentage
Positive	39	75.00
Negative	6	11.50
Non-determinable	7	13.50
Total	52	100

Among 39 *MBL* positive isolates, the in-vitro antibiotic susceptibility results are given as count and percentages were; piperacillin/ tazobactam in 12 (30.8%) cases, amikacin 7 (17.9%), polymyxin B 7 (17.9%), tobramycin 5 (12.8%), cefoperazone/ sulbactam and ceftazidime each in 2 (5.1%), ciprofloxacin, moxifloxacin, colistin sulphate, tetracycline, azithromycin and aztreonam each in 1 (2.6%), co-trimoxzole, gentamicin and rifampin each in zero cases.

When ß-lactams (except aztreonam) and ß-lactam/ ß-lactam inhibitor combination (piperacillin/ tazobactam, cefoperazone/ sulbactam and ceftazidime) are excluded because all of them are hydrolyzed by *MBL*. We are left with 14 isolates out of 39 (35.8%) showing sensitivity to one or more than one antibiotics. The susceptibility pattern of these *MBL* positive isolates are shown in Table 5.

Table 5: MBL positive on Criteria 1A (14/39); details of one, two and three antibiotic regimes (excluding TZP, SCZ & CAZ)

Sr. No	CIP	MXF	AK	Tob	PB	CT	TET	AZM	ATM
Total	1	1	7	5	7	1	1	1	1
% age	2.6	2.6	17.9	12.8	17.9	2.6	2.6	2.6	2.6

Key: CIP (Ciprofloxacin), MXF (Moxifloxacin), AK (Amikacin), Tob (Tobramycin), PB (Polymyxin B), CT (Cholistine), TET (Tetracyclin), AZM (Azithromycin), ATM Aztreonam)

DISCUSSION

Data from various parts of the world show several criteria for *MBL* screening. All these methods depend upon the fact that *MBL* activity is inhibited by chelating agents like EDTA.[7]

These methods include modified Hodge test, double disc synergy test using Imipenem-EDTA discs or ceftazidime-EDTA discs, EDTA-impregnated Imipenem disc and EDTA impregnated meropenem discs and Imipenem-EDTA impregnated E-test strips.[8] We used the disc potentiating test with Imipenem-EDTA discs. This is the most simple, highly sensitive and specific method employed in a number of widely published studies.[9] However, our study was hindered by the fact that EDTA itself shows antibacterial activity. (Table 3)

To overcome the undesirable disadvantage of EDTA, we placed all those isolates of Imipenem resistant *P. aeruginosa* which showed zone of inhibition to EDTA alone of ≥ 16 mm (the breakpoint inhibition zone for Imipenem sensitivity) in the non-determinable category. As such we employed modified Criteria 1A to overcome to some extent our false positive results. Moreover, following this criteria, our results are comparable with those published elsewhere in the world.[10]

As per Criteria 1A, 75% of our Imipenem resistant isolates tested positive for *MBL* and only 11.5% were *MBL* negative. We concluded that the 13.5% of Imipenem resistant isolates in the non-determinable category should also be treated positive for *MBL* for clinical purposes. Moreover, genotypic confirmation (like PCR) was not part of our study and so presence of this gene in the non-determinable isolates could not be ruled out.

Our study reflects that once an isolate is declared *MBL* positive, the room for antibiotic therapy is highly compromised. Keeping in view that *MBLs* hydrolyses all ß-lactam antibiotics as well as serine ß-lactamase inhibitors (like clavulanic acid), the treatment options are severely limited. Moreover, genes encoding *MBLs* production are clustered with those encoding resistance to aminoglycosides and fluoroquinolones, thus further compromising our antibiotic regimen policy for these isolates.[11,12]

In this study we found that only 14 among the 39 (35.8%) *MBL* positive isolates showed susceptibility to one or more than one antibiotics. As monotherapy for *MBL* positive isolates is usually unsuccessful. As such we have only 6 (15.9%) isolates showing sensitivity to two or three antibiotics with different mechanisms of action. Only in these patients with *MBL* positive strains, a successful therapeutic regimen was possible.

Aztreonam is the sole ß-lactam which is resistant to the hydrolytic action of *MBL*. However its wide spread use and susceptibility to extended-spectrum beta-lactamase

(ESBL) have greatly limited its activity as has been reported in this study.[12] We suggest that this antibiotic should be strongly contraindicated for the treatment of infections other than those caused by *MBL* positive *P. aeruginosa*.

The present study looks at the predominant determinants of Imipenem resistance and the main sites of infection and colonization by this organism. It has been noted that there is an increasing incidence of Imipenem resistance in *P. aeruginosa* with resistance rate as high as 60%.[13]

The main factor behind this resistance is *MBL*. Its presence in *P. aeruginosa* and the ease with which it spreads among different gram negative bacilli is a major clinical and public health problem and poses a challenge to antimicrobial therapy.[14]

In this study, we also tried to establish the sensitivity pattern of Imipenem resistant *P. aeruginosa* to non-conventional antipseudomonal agents like Tetracycline, Co-Trimoxazole, Rifampin etc but their activity against these isolates too, was very poor. Lipopeptides (Polymyxin B and colistin) have been claimed to be effective alternatives.[15] Moreover, these antibiotics are costly and not easily available and are contraindicated as monotherapy due to rapid in vivo acquisition of resistance.[16]

It is urgently needed that ESBL should be properly detected so as to avoid false positive results and restrict the use of carbapenem. This group of antibiotics may be the main force behind the emergence of *MBL* positive isolates. Similarly Glycopeptides (vancomycin and Teicoplanin) are the other notorious agents leading to the emergence of *MBLs*.[17] The accurate identification and reporting of *MBL* producing *P. aeruginosa* will aid infection control practitioners in preventing the spread of these multidrug-resistant isolates.[8] Our results support the notion that clinical microbiology laboratories must be able to distinguish *MBL* producing *P. aeruginosa* from strains with other mechanisms of resistance. In the absence of novel agents for the treatment of infections caused by multidrug-resistant gram negative bacteria in the near future, the uncontrolled spread of *MBL* producers may lead to treatment failures.[11]

We concluded that *MBL* production seems to be the most important emerging resistance mechanism in *P. aeruginosa*. An early detection of *MBL* producing *P. aeruginosa* may avoid the future spread of these multidrug-resistant isolates. We recommend that all Imipenem resistant. *P. aeruginosa* isolates be routinely screened for *MBL* production using the IMP-EDTA disc screen test as described in this study. These steps will ensure optimal patient care and the timely introduction of appropriate infection control procedures.

To control the problem of multi drug resistant organisms in any hospitalized infections, measures should be directed towards continuously monitoring the presence of these organisms, and the avoidance of excessive and continual use of any single agent over a long period of time.

CONCLUSION

MBL production in *P. aeruginosa* confers a challenge

for clinicians to treat such resistant infections with conventional antibiotics. Therefore testing each Imipenem resistant Pseudomnas aeruginosa for *MBL* production must be taken in routine consideration.

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CONFLICT OF INTEREST
Authors declare no conflict of interest.
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AUTHORS' CONTRIBUTION

The following authors have made substantial contributions to the manuscript as under:

Conception or Design:	FR, HK
Acquisition, Analysis or Interpretation of Data:	FR, SA, HK
Manuscript Writing & Approval:	FR, SA, HK

All the authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.