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ORIGINAL ARTICLE

Distribution of Extended-spectrum β-lactamase and Metallo-β-lactamase-producing *Pseudomonas aeruginosa* in Tertiary Care Hospitals of Lahore, Pakistan

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ABSTRACT

Background: Pseudomonas aeruginosa (*P. aeruginosa*) is an important bacterial pathogen most frequently associated with nosocomial infections, especially in immuno-compromised patients. Early detection of these life threatening, β-lactamase producing bacteria is essential for infection control and to prevent their dissemination. The aim of our study was to detect the presence of Extended-Spectrum β-Lactamase (ESBL) and Metallo-β-Lactamase (MBL) strains of *Pseudomonas aeruginosa*.

Material and Methods: Eighty-eight identified strains of *P. aeruginosa* were collected from Chughtai Laboratories, Combined Military Hospital and Children Hospital, Lahore. These strains were sub-cultured and after confirming the cultural characteristics by Gram staining and colony morphology, manual biochemical identification was done. Susceptibility to various antibiotics and production of extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs) were determined using modified Kirby Bauer disk diffusion method, double disk synergy test, combined disk synergy test (CDST) and inhibitor-potentiated disk diffusion test (IPD) respectively.

Results: Out of eighty-eight strains tested, three were ESBL producers (3.4%) and eleven strains (12.5%) were found to be resistant to carbapenems. Of these, eight were MBL producers (72.7%). All these β-lactamase producing strains (14 strains) were multidrug-resistant (MDR). Piperacillin and piperacillin/tazobactam proved to be the most effective antibiotics in both types of β-lactamase producing strains.

Conclusion: Our study shows noticeable emergence of β-lactamases (ESBLs & MBLs) in *P. aeruginosa*. All of these strains were MDR. It reveals a correlation of these β-lactamases with multidrug resistant genes.

Key words: ESBL, MBL, MDR. *Pseudomonas aeruginosa,* Pakistan, DDST, CDST, IPD

Authors' Contribution:
[1,2] Conception, synthesis, planning of research and manuscript writing [3-5] Interpretation, discussion, [6,7]Data analysis, Active participation in data collection.

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Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen responsible for various healthcare associated infections like pneumonia, sepsis, wounds and urinary tract infections.[1,2] This organism can cause deadly infections and is most commonly isolated from wound infections in developing countries.[3,4] It is professed to be associated with high mortality rate i.e. up to 61%.[5] Carbapenems are most effective antibiotics against

several pseudomonal infections. However resistance to this innovative antibiotic has been observed in recent years.[6] Metallo β-lactamase is usually associated with carbapenems-resistance in *P. aeruginosa*.[7] MBL hydrolyzes most of the β-lactam antibiotics except monobactams. Additionally, these enzymes are resistant to most of the β-lactam inhibitors like clavulanic acid, sulbactam.[8] Moreover, MBL-producing *P. aeruginosa* are responsible for high a mortality rate.[9]

Pseudomonal infections are often burdensome because of an intrinsic and acquired resistance of the organism to common antimicrobials, eventually resulting in emergence of multidrug resistant strains of *P. aeruginosa*.[10] Among these different resistant mechanisms, β-lactamases including Extended-Spectrum β-Lactamases and Metallo β-Lactamases are predominantly observed in *P. aeruginosa*.[11] ESBL hydrolyzes β-lactam drugs like cefotaxime, ceftriaxone, ceftazidime and monobactams with no efficacy on cephamycins and carbapenems. β-lactamase inhibitors like clavulanic acid are effective against these enzymes.[8,12]

The aim of this research was to identify ESBL and MBL-producing *P. aeruginosa* and to determine the antimicrobial susceptibility patterns of these strains (ESBL and MBL producing *P. aeruginosa*).

Material and Methods

The study was conducted at Department of Microbiology, University of Health Sciences, Lahore. This was an observational, cross-sectional study conducted over a duration of one year from October 2008 to October 2009. Eighty-eight strains of *P. aeruginosa* were collected from Chughtai Lahore Laboratories, Combined Military Hospital, Lahore and Children Hospital, Lahore, where these strains were isolated from wound swabs, pus, bronchial washings and blood. Identified strains of *P. aeruginosa* were sub-cultured in Department of Microbiology, University of Health Sciences, Lahore. After confirming the cultural characteristics by Gram staining and colony morphology, manual biochemical identification was done by API 20NE identification system (BioMerieux, France). Bio-statistical analysis was done by Pearson's chi-square test as previously used by Giriyapur et al.[13]

Antimicrobial susceptibility of *P.aeruginosa* was performed using Mueller-Hinton agar (Oxoid UK), according to Clinical Laboratory Standards Institute (CLSI, 2009) guidelines. Antibiogram profile was generated by using: amoxicillin/clavulanic acid (30μg), ceftriaxone (30μg), ceftazidime (30μg), ciprofloxacin (5μg), sulfamethoxazole/trimethoprim (25μg), piperacillin (100μg), piperacillin/tazobactam (100\10μg), aztreonam (30μg), meropenem (10μg), imipenem (10μg), and amikacin (30μg). Amoxicillin/clavulanic acid was used for screening of ESBL producers and sulfamethoxazole/trimethoprim (SXT 5μg) were used to check whether it is effective in β-lactamase producers (Figure 1).

ESBL production in all the isolates was detected by double disc synergy test (DDST) as described byJarlier et al.[14] Synergistic effect of amoxicillin + clavulanic acid (20 + 10 µg) was checked with ceftazidime (30 µg) and ceftriaxone (30 µg). Strains indicating >5mm synergistic zone were confirmed as ESBLproducers.[15]

MBL production in the carbapenem-resistant isolates was detected by following two methods. *Pseudomonas aeruginosa* and *Enterobacter cloacae* positive for MBL were used as positive control. For combination disc test (CDST), imipenem (10 µg) and meropenem (10 µg) discs (Oxoid) alone and in combination with 0.5 M EDTA were used. Increase in the inhibition zone of ≥ 7mm by the addition of EDTA indicates MBL-production.[16] For Inhibitor potentiated disk diffusion test (IPD), imipenem (10 µg) (Oxoid) was used along with disc of 0.5 EDTA solution. Presence of an augmentation zone (clearing zone) i.e. >7mm between EDTA and imipenem discs was interpreted as a positive test.[17].

Results

Antimicrobial susceptibility pattern of *P. aeruginosa* strains (Table I) showed piperacillin/tazobactam as the most sensitive antibiotic with 95.5% susceptible isolates. Piperacillin (94.3%) was second most sensitive antibiotic. There was no significant difference between these two antibiotics. It was followed by meropenem (89.8%), imipenem (87.5%), amikacin (84.1%), ceftazidime (80.7%), aztreonam (71.6%), and ciprofloxacin (69.3%).

Ceftriaxone was least effective among β- lactams with only 29.5% susceptible isolates. Amoxicillin/clavulanic acid and co-trimoxazole were resistant in all isolates (Figure 1).

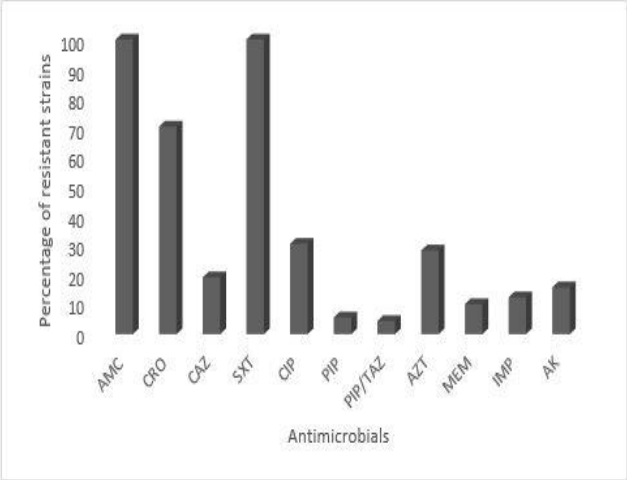

Figure 1: Antimicrobial resistance pattern of P. aeruginosa strains (n=88). Here, AMC =amoxicillin/calvulonic acid, CRO =ceftriaxone, CAZ =Ceftazidime, SXT=sulphamethoxazole/trimethoprim, ATM aztreonam, AK =Amikacin, CIP = ciprofloxacin, PRL= piperacillin, TZP=piperacillin/tazobactam, IPM=imipenem and MEM= Meropenem.

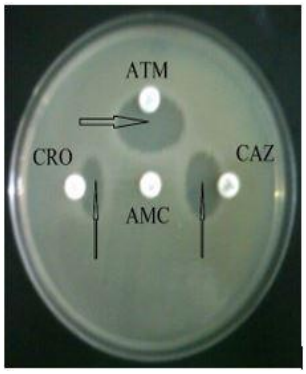

Figure 2: Demonstration of ESBL phenomenon by Double Disc Synergy test (DDST)

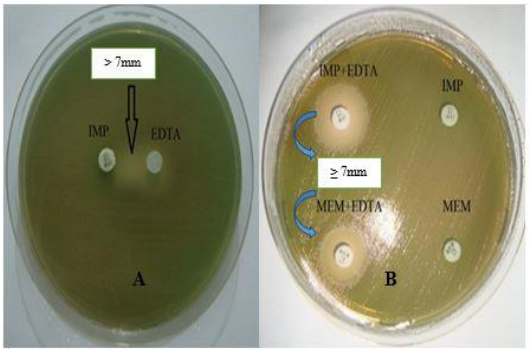

Figure 3: MBL detection tests. A) Combined Disk Synergy Test and B) Inhibitor Potentiated Disk Diffusion

Table: I. Antimicrobial susceptibility pattern of P. aeruginosa isolates (n=88)

Sr. No	Antimicrobials	NS	S (%)	NR	R (%)
1	Co-amoxiclav	0	0	88	100
2	Ceftriaxone	26	29.5	62	70.5
3	Ceftazidime	71	80.7	17	19.3
4	Cotrimoxazole	0	0	88	100
5	Ciprofloxacin	61	69.3	27	30.7
6	Piperacillin	83	94.3	5	5.7
7	Piperacillin/tazobactam	84	95.5	4	4.5
8	Aztreonam	63	71.6	25	28.4
9	Meropenem	79	89.8	9	10.2
10	Imipenem	77	87.5	11	12.5
11	Amikacin	74	84.1	14	15.9

n =Total number of strains
NS=number of sensitive strains
NR= number of resistant strains
S (%) = percentage of sensitive strains
R (%) = percentage of Resistant strains

Table II: Antimicrobial resistance pattern of ESBL and MBL-producing strains of Pseudomonas aeruginosa

Sr. No	Antibiotics	ENR	E (%) R	MNR	M (%) R
1.	Co-amoxiclav	3	100	8	100
2.	Ceftriaxone	3	100	8	100
3.	Ceftazidime	3	100	8	100
4.	Cotrimoxazole	3	100	8	100
5.	Ciprofloxacin	3	100	8	100
6.	Piperacillin	0	0.0	0	0.0
7.	Piperacillin/tazobactam	0	0.0	0	0.0
8.	Aztreonam	3	100	6	75
9.	Meropenem	0	0.0	8	100
10.	Imipenem	0	0.0	8	100
11.	Amikacin	2	66.6	8	100

ENR= number of resistant strains among ESBL producers
E (%) R = percentageof resistant strains among ESBL producers
MNR=number of resistant strains among MBL producers
M (%) R =percentageof resistant strains among MBL producers

Out of 88 cultured isolates of *P. aeruginosa* three (3.4%) were ESBL-producers and eleven strains (12.5%) were resistant to carbapenems of which eight (72.7%) were MBL-producers. All the ESBL and MBL-producing strains were found to be MDR. ESBLs were resistant to β-lactam antibiotics except carbapenems where 100% susceptibility towards these antibiotics was observed.

Moreover, ESBLs also indicated high susceptibility towards amikacin (Table II). MBL-producers indicated 100% resistance towards applied antibiotics except piperacillin and piperacillin /tazobactam combination where 100% sensitivity was observed (Table III).

Table III. Antimicrobial susceptibility pattern of MBL-producing and Non-producing Isolates

Antimicrobials	MBL Producing (n = 08)			MBL Non-producing (n = 80)			X2
	R	S	S (%)	R	S	S (%)	*P* Value
Co-amoxiclav	8	0	0.0	80	0	0.0	*
Ceftriaxone	8	0	0.0	54	26	29.5	0.05
Ceftazidime	8	0	0.0	09	71	80.7	0.00
Cotrimoxazole	8	0	0.0	80	0	0.0	*
Ciprofloxacin	8	0	0.0	19	61	69.3	0.00
Piperacillin	0	8	100	05	75	94.3	0.467
Piperacillin/ tazobactam	0	8	100	04	76	95.5	0.517
Aztreonam	6	2	25	19	61	71.6	0.02
Meropenem	8	0	0.0	01	79	89.8	0.00
Imipenem	8	0	0.0	03	77	87.5	0.00
Amikacin	8	0	0.0	06	74	84.1	0.00

R= Resistant

S= Sensitive

** = no statistics is computed as AMC and SXT are constant (Resistant in all isolates).*

P value < 0.05 = significant difference

Discussion

P. aeruginosa is an important nosocomial pathogen, endowed with a variety of resistance mechanisms that may cause multidrug or even pan-drug resistance. Extended-spectrum β-lactamases (ESBLs) and carbapenemases (MBLs) are among the most common causative agents.[18] In the present study, three strains (3.5%) were ESBL producers detected by the double disc synergy test which is supported by the results of Kotwal *et al* in which 6% of ESBL were detected among cefepime resistant *P. aeruginos.*[19,20] While the findings of Wolska and Jakubczak, (2008) showed no ESBL detection in *P. aeruginosa* isolates.[21] However, it is in contrast to the study conducted in Pakistan, where 35.8% strains of *P.aeruginosa* were ESBL-producers.[22] This disparity might be due to the evidence that more MDRs are isolated from burn units.[23]

In the present study eleven strains (12.5%) of *P. aeruginosa* indicated resistance to carbapenems of which eight were detected as MBL-producers by using the CDST and IPD methods. Our data indicates that frequency of MBL-producing strains among imipenem resistant *P. aeruginosa* is 72.7%. While Irfan *et al* reported 100% of MBL-production among carbapenem resistant *P. aeruginosa*.[24] Our study results are similar to the findings of Kali *et al* where 72.7% MBL-producers among carbapenem-resistant *P. aeruginosa* isolates were observed.[25] A recent study in Pakistan has described the incidence of ESBL and MBL in clinical isolates of MDR *P. aeruginosa* as 23.94% and 40.84% respectively.[26]

Our data showed increased resistance to commonly used antibiotics. Piperacillin/tazobactam and piperacillin alone proved to be effective antibiotics. Carbapenems were found to be the second most effective antibiotic group accounting for 12.5% and 10.2% resistance for imipenem and meropenem respectively, which is consistent with national antibiotic resistance data of Pakistan in 2009.[27]

The β-lactamase-producers were resistant to all other antibiotics except the above-mentioned ones, so there was a narrow range for a suitable drug of choice. *P. aeruginosa* had shown an increased resistance to the fluoroquinolone (30.7%). Resistance rates of amikacin, ceftazidime and aztreonam remained 15.9%, 19.3%, 28.4% respectively and similar reports of 22%, 30% and 19% resistance have been reported by Pakistan Antimicrobial Resistance Network (PARN). Ceftriaxone was least effective among β-lactams with only 29.5% susceptible isolates. All isolates were resistant to amoxicillin/clavulanic acid and co-trimoxazole (as already established). These values are comparable to the findings available in Pakistan that are 83.8% and 79.24% resistance respectively.[22] There were 14 (15.9%) isolates as MDR, three of these were ESBL and eight out of twelve carbapenem resistant isolates were MBL-producers. This is an alarming sign as few therapeutic options are left for the patients infected with these strains.

Early screening of *P. aeruginosa* isolates to detect ESBL and MBL-production should be emphasized. Therefore, routine testing of the isolates of *P. aeruginosa* for sensitivity to ceftazidime, cefotaxime and carbapenems may represent a cost-effective way for screening of

ESBLs and MBLs. Our study has introduced an easy and cost-effective inhibitor potentiated disk diffusion (IPD) method for MBL detection in Pakistan. Thus, double disk synergy test and combined disk synergy test (CDST) / inhibitor-potentiated disk diffusion method (IPD) can easily be used to confirm the ESBL and MBL phenotypically.

The emergence of these β-lactamases along with MDR genes in *P. aeruginosa* may adversely muddle the clinical management of such patients. High frequency of these enzymes urges the infection control teams of hospitals to design some preventive measures to stop the dissemination of these resistant strains.

Conclusion

Our study shows noticeable emergence of these β-lactamases in *P. aeruginosa*. All of these strains were MDR. It reveals a correlation of these β-lactamases with multidrug resistant genes.

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