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Concomitant Detection of Biofilm Formation and MBL Production in Meropenem Resistant Isolates of *Pseudomonas aeruginosa*

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Authors' contributions

This work was carried out in collaboration between all authors. Author SS participated in study design, data collection, data analysis, and wrote the first draft of the manuscript and managed literature searches and manuscript revisions. Authors GB and RG participated in study conception, design and data collection. All authors managed the analysis of the study, read and approved the final manuscript.

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ABSTRACT

The purpose of this study was to detect biofilm formation and to examine the correlation between biofilm and Metallo-β-lactamases (MBL) production in *Pseudomonas aeruginosa.* A total of 64 *P. aeruginosa* isolates were identified using standard microbiological methods and antimicrobial susceptibility testing (AST) was performed on them according to Clinical and Laboratory Standards Institute (CLSI) guidelines. The isolates were screened for biofilm production using both qualitative and quantitative methods. The presence of MBL genes were checked by multiplex PCR assay. Out of all 30 meropenem resistant *P. aeruginosa*, 2 isolates were found producing all the three genes (i.e. bla_{IMP}, bla_{VIM}, bla_{SIM}) for MBL production and they were found to produce biofilm. Resistant to four antibiotics such as aztreonam (85.7% vs 11.1%, *P*< 0.000), Cefepime (82.1% vs 2.8%, *P*<0.000) gentamycin (82.1% vs 27.8%, *P*< 0.000) and Pipercillin/Tazobactum was also high

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(28.6% vs 2.8% *P*< 0.003) was comparatively higher among biofilm producers than non biofilm producers. In biofilm production, both qualitative method and quantitative plate method showed 16 isolates (53.3%) as biofilm producers for MBL genes. Out of these 16, only 9 isolates showed MBL production along with biofilm production having significant association (*P*<0.004).
The prevalence of MBLs has been increasing worldwide, particularly among *P. aeruginosa*, leading to severe limitations in the therapeutic options for the management. Presence of MBL genes has a role in inducing biofilm production and significant association in *P. aeruginosa* isolates. Overall, drug resistance was found to be more in biofilm producing isolates than non biofilm isolates.

Keywords: P. aeruginosa; MBL; biofilm formation.

1. INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen and a major cause of nosocomial infections and is also considered as most challenging pathogen globally because of its high rate of resistance to antimicrobial agents [1].

P. aeruginosa is reported to be amongst the leading causes of nosocomial infections. It is known to exhibit intrinsic resistance to several antimicrobial agents. MBLs are class B enzymes which hydrolyze carbapenems and are encoded by genes like IMP (Imipenemase), VIM (Verona integrin-encoded metallo-β-lactamase), etc. [2].

Reserpine a plant alkaloid has been used to inactivate MDR efflux pump. Efflux pumps are transport proteins involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment. In the case of *P. aeruginosa,* this has traditionally been attributed to the presence of a highly impermeable outer membrane [3].

Resistance to various antibiotics and substances with antimicrobial activity has been associated with bacterial biofilm formation and phagocytosis by components of the adaptive immune system as well as various nosocomial infections caused by *P. aeruginosa*.

Therefore the present study was undertaken to determine the correlation between frequency of biofilm formation in relation to MBL production.

2. MATERIALS AND METHODS

This is a cross sectional study conducted at the Department of Pulmonary Medicine and Microbiology department at a tertiary care hospital in Lucknow, from between September 2010 to August 2012. A total of 200 sputum and Bronco alveolar lavage (BAL) samples of LRTI patients were taken in which 64 *P. aeruginosa* were identified according to the standard procedure and antibiotic susceptibility test was done by Kirby Bauer disk diffusion method as per CLSI 2010 guidelines [4] MDR strain were classified as MDR if they were resistant to ≥3 classes of antibiotics.

Minimum inhibitory concentration (MIC) was determined for *P. aeruginosa* strains resistant to meropenem by the agar dilution method [4]. Dilutions of meropenem ranging from 2 µg/ml to 128 µg/ml was prepared in doubles. MIC of ≥16 µg/ml was interpreted as resistant. Meropenem powder was obtained from Himedia (Mumbai), India. ATCC *P. aeruginosa* 27853 was used as control.

All Meropenem resistant strains tested for MIC were simultaneously tested for efflux mechanism. In this test, reserpine is used as an inhibitor of efflux pump resulting in increased sensitivity towards drug and thereby reducing MIC. Double dilutions of meropenem with and without reserpine (25 and 50 µg/mL) were prepared by broth dilution method. Decrease in MIC of plates with reserpine compared to plates without reserpine was considered positive for efflux mechanism [5].

Meropenem resistant *P. aeruginosa* isolates were further analyzed for MBL production by double disc synergy test, Hodge test [6] and combined disk test [7].

A multiplex PCR assay was performed to detect and differentiate three families of acquired MBL encoding genes (VIM, IMP, and SIM families) in a single reaction [8].

2.1 Tube Method (TM) and Tissue Culture Plate (TCP) Method

The Qualitative biofilm formation assay and quantitative biofilm formation test was carried out

as described by M. Dheepa et al. [9] with some modifications. The experiment was performed three times, and the results were averaged. *P. aeruginosa* PAO1 (Biofilm producing) and *P. aeruginosa* ATCC 27853 (non-biofilm-producing) were used as positive and negative controls in both test.

2.2 Statistical Analysis

Statistical analysis was performed wherein categorical variables were compared using Chi-square test. Differences were significant if the *P*-value associated with the test was < 0.05.

3. RESULTS

Among the 200 total clinical samples, 64 (32%) isolates of *P. aeruginosa* were identified. majority of *P. aeruginosa* (70.3%) was isolated From Sputum samples, followed by BAL sample where 29.7% *P. aeruginosa* was isolated. Interestingly, it was observed that 53.5% of isolates among the MDR. These isolates were strongly positive for biofilm.

MIC for meropenem resistant isolates ranged from 2-128 µg/ml with 50% isolates showing MIC in resistance zone *ie*; >16 µg/ml. Three isolates having MIC ≥32 µg/ml were all found to be biofilm producers. The presence of reserpine inhibited MDR efflux pump in 6 (20%) isolates of *P. aeruginosa* whereas 80% showed absence of efflux pump.

Thirty meropenem resistant isolates were screened for MBL production in which 9 isolates exhibited more than 7 mm zone size enhancement by the combined disc method whereas only 16 gave positive result by DDST but none were positive by MHT. Genotypic analysis was positive for 16 of the isolates. bla_{VIM} gene was present in 9 isolates, 4 were bla_{SIM} gene positive and 3 had bla_{IMP} gene.

Among 64 isolates, biofilm production by qualitative tube method was strongly positive for 16 (25%) isolates and 48 (75%) isolates were weakly adherent. In tissue culture plate method, biofilm formation was strongly positive in (19%), moderate in (23.4%) and weak or no biofilm production was seen in 37 isolates (58%). Almost all the multiple antibiotic resistant isolates tested for biofilm formation.

The overall percentage of resistance observed among all the *P. aeruginosa* isolates including biofilm producers and biofilm non-producers for 14 antibiotics tested, is given in Table 1. This table shows that the resistance of antibiotic is more for most of the strongly biofilm positive isolates compared to the biofilm negative isolates. Isolates showed maximum resistance to ampicillin and amoxyclav 97%, levofloxacin 78.2%, 66% ceftazidime and ceftriaxone. Imipenem was found to be more effective antibiotics. However, resistance to other four antibiotics such as aztreonam (85.7% vs 11.1%, $P< 0.000$), Cefepime (82.1% vs 2.8%, $P<0.000$) gentamycin (82.1% vs 27.8%, $P< 0.000$) was comparatively higher among biofilm producers than non-biofilm producers. Resistance among biofilm producers to Pipercillin/Tazobactum was also high (28.6% vs 2.8% $P< 0.003$) when compared with non biofilm producers isolates. Least resistance (7.1%) was noticed only for imipenem.

Out of 16 (53.3%) biofilm producing isolates, only 8 showed presence of MBL genes. Two isolates carried all the three genes, 2 isolates carried bla_{VIM}, bla_{SIM} genes and four isolate had only bla_{VIM}, remaning 8 isolates were negative for MBL genes. The association between biofilm production and MBL genes was found to be highly significant with $P< 0.004$ (56.2 *vs* 43.7).

Biofilm positive isolates were found to become more resistant then biofilm negative isolates (Table 1).

4. DISCUSSION

P. aeruginosa is a well-recognized nosocomial pathogen that can cause severe infections in hospitalized patients. An important characteristic of these bacteria is its natural resistance to different antibiotics and their ability to horizontally acquire genetic material that promotes genetic exchange among intrahospital species [10].

In the present study, the antibiogram of the 64 isolates of *P. aeruginosa* showed more resistance to gentamicin, amikacin, ceftazidime, tobramycin, ceftriaxone which was in concordance with the findings of other studies [11].

In a few Indian studies, the rate of carbapenem resistant in *P. aeruginosa* has been reported to vary from 12-37% [12].

In the present study *P. aeruginosa* isolates were more sensitive to imipenem than to meropenem. Whereas in another study by Alicia et al. [13] reported higher resistant rate of meropenem was found in *P. aeuginosa* isolates.

Table 1. Antibiotic resistance pattern of the *P. aeruginosa* isolates

Antibiotics	Resistance		
	Biofilm positive isolates (n=28)	Biofilm negative isolates (n=36)	Resistance of all (n=64)
Ampicillin	27 (96.4)	35 (97.2)	96.8
Amikacin	11 (39.2)	8 (22.2)	29.6
Amoxiclav	27 (96.4)	35 (97.2)	96.8
Aztreonam	24 (85.7)	4 (11.1)	43.7
Ciprofloxacin	6 (21.4)	9 (25)	23.5
Ceftazidime	18 (64.2)	24 (66.7)	65.6
Cefepime	23 (82.1)	1 (2.8)	37.5
Ceftriaxone	18 (64.2)	24 (66.7)	65.6
Gentamicin	23 (82.1)	10 (27.8)	51.6
Imipenem	2 (7.14)	0 (0)	3.13
Meropenem	12 (42.9)	18 (50)	46.8
Pipercillin/Tazobactum	8 (28.6)	1 (2.8)	14.1
Tobramycin	10 (35.7)	9 (25)	29.6
Levofloxacin	22 (78.5)	28 (77.8)	78.2

**All percentages are given in brackets*

Reserpine has been shown not to inhibit all types of efflux pumps. These may partly explain the absence of reserpine MDR inhibited efflux pumps in some strains of *P. aeruginosa.* The other possible reasons include absence of reserpine inhibited pumps, and for the resistant strains, resistance may be due to other mechanisms i.e.; decrease outer membrane permeability, mutation resulting in altered target site with less affinity for the drugs.

Carbapenems are the drugs of choice for infections caused by *P. aeruginosa*. In recent years, in several countries (Africa, Europe, Mexico, Central and South America) an increase in *P. aeruginosa* strains resistant to carbapenems has been observed, which has generated a health problem of great interest for therapeutic treatments [10]. The resistance to carbapenems, especially in *P. aeruginosa*, results from reduced levels of drug accumulation or increased expression of pump efflux or production of MBL [14-16].

Overuse of carbapenems (imipenem and meropenem) in the treatment of nosocomial infections caused by strains of *P. aeruginosa* has facilitated the emergence of an elevated resistance to these antibiotics.

In the studies by Kumar et al. [17] in nosocomial *P. aeruginosa* isolates in India, it was concluded that the high prevalence of *P. aeruginosa* strains resistant to carbapenems and MBL producers was due to the excessive use of carbapenems in hospitals when treating nosocomial infections.

Based on the findings of our study we found that the EDTA disk synergy test was better than modified Hodge test for detection of carbapenemase and MBL in our setting. CLSI also recommends MHT for detection of carbapenemases activity in enterobacterecia only this could also be the reason for non detection of MBL by MHT.

This present study investigated the predominant β-latamase coding genes such as, bla_{VIM}, bla_{IMP}, and bla_{SIM} through multiplex PCR. Among MBL producing isolates in our study, the presence of bla_{VIM} gene is predominant when compared with bla_{IMP} and bla_{SIM} MBL gene. The presence of bla_{VIM} gene appears to be more prevalent in our setup, as 30% of isolates were positive for bla_{VIM} MBL. This finding suggests successful global dissemination of bla_{VIM} resistant gene that is of great concern. This bla_{VIM} gene was found to be more prevalent among MDR *P. aeruginosa* isolates in our setup as revealed by multiplex PCR method.

Additionally, production of biofilms by *P. aeruginosa* makes intrahospital infections complicated to treat due to its highly organized structure, which functions as a barrier for antimicrobial action and production of MBL as a known and causative factor of resistance in nosocomial strains. Bacteria within the biofilms are more resistant to physical and chemical changes by different chemotherapeutic agents than bacteria in their platonic growth stage [10].

Biofilm forming isolates were also less frequently resistant to imipenem and our results show

53.5% multi-drug resistant isolates were strongly positive for biofilm producers.

In our study compared to non biofilm producers, isolates producing biofilms significantly revealed higher resistance to cefepime, gentamicin, aztreonam and piperacillin / tazobactam.In this study 56.2% isolates carried MBL genes with biofilm formation indicating that there is a strong relationship between biofilm formation and the production of MBL genes. Since we have only seven strong biofilm forming isolates were positive for MBL genes. Interestingly, rest of the MBL genes positive isolates (n=1) were weakly adherent or produced negligible biofilm.

5. CONCLUSIONS

This study demonstrates high propensity among the clinical isolates of *P. aeruginosa* to form biofilm and a strong significant association of biofilm with MBL genes. The concurrent detection of biofilm production and various resistance mechanisms among beta-lactam resistant *P. aeruginosa* may help in designing newer therapies based on interference with biofilm formation and thus, countering clinical episodes of antibiotic resistance.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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