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Correlation of Biofilm Production in MBL-producing Pseudomonas aeruginosa isolates at a Tertiary Care Hospital.

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Abstract

Pseudomonas aeruginosa is a leading cause of hospital-acquired infections, with high morbidity and mortality, especially in immunocompromised patients. The production of biofilms and the presence of metallo-β-lactamases (MBLs) contribute significantly to its antimicrobial resistance and persistence in clinical settings. MBL-producing P. aeruginosa exhibits resistance to carbapenems, making infections difficult to treat. This study explores the correlation between biofilm formation and MBL production in clinical isolates from a tertiary care hospital.

Material and methods- The present study was conducted in the department of Microbiology, GMC, Kota (Rajasthan), India. 225 non-duplicate isolates of *Pseudomonas aeruginosa* from various clinical samples such as pus, urine, sputum, ET and body fluids were taken for the study.

All isolates were subjected to routine antibiotic susceptibility testing by Kirby Bauer Disc Diffusion method. Metallo-β-Lactamases (MBLs) were phenotypically detected by Modified Hodge test and Biofilm production by Microtiter plate method.

Result- Out of 225 sample, maximum number of isolates were obtained from sputum 68 (30%), followed by urine 59 (26%), pus 57 (25%), body fluids 26 (12%), and endotracheal tube 15 (7%). Out of 62 Biofilm producers, 41 (66.1%) were MBL and 9(14%) were non-producers. Biofilm producing isolates showed more resistance as compared to non-biofilm producers. The observed difference between biofilm formation for multidrug resistance and susceptible isolates was found to be statistically significant.

Conclusion: Regular surveillance of resistance patterns and biofilm-forming capabilities of *P. aeruginosa* is crucial for guiding appropriate antimicrobial therapy. This study provides critical insights into the epidemiology of MBL production and biofilm formation in a tertiary care setting, underscoring the need for enhanced diagnostic and therapeutic approaches.

Keywords: *Pseudomonas aeruginosa*, Metallo-β-lactamase (MBL), biofilm, antimicrobial resistance, tertiary care hospital

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen that ranks among the most common causes of hospital-acquired infections (HAIs). It is particularly notorious for causing infections in immunocompromised individuals, such as those with burns, cancer, cystic fibrosis, or undergoing invasive medical procedures. The organism's pathogenicity is driven by its intrinsic resistance to multiple antibiotics, ability to acquire additional resistance mechanisms, and capacity to form biofilms on both biotic and abiotic surfaces.[1,2]

Metallo-β-lactamases (MBLs) are one of the most critical resistance mechanisms in *P. aeruginosa*. These enzymes hydrolyse a broad range of β-lactam antibiotics, including carbapenems, which are often considered the last line of defence against multidrug-resistant Gram-negative infections. The rapid dissemination of MBL-producing isolates in healthcare settings has become a significant public health concern, necessitating continuous monitoring and effective infection control strategies.[3]

Biofilm formation further complicates the treatment of *P. aeruginosa* infections. Biofilms are structured communities of bacterial cells encased in an extracellular polymeric substance (EPS) that adhere to surfaces. These structures provide bacteria with protection against antimicrobial agents and host immune responses, leading to chronic and recurrent infections. The interplay between MBL production and biofilm formation creates a formidable challenge in managing *P. aeruginosa* infections.[4]

Understanding the correlation between MBL production and biofilm formation in *P. aeruginosa* isolates is crucial for developing targeted therapeutic strategies and informing infection control policies. This study aims to analyse the MBL profile and biofilm-forming capabilities of *P. aeruginosa* isolates obtained from various clinical samples in a tertiary care hospital, providing insights into their role in antimicrobial resistance and patient outcomes.

MATERIAL AND METHODS

1.Sample Collection and Identification:

Isolated Pseudomonas aeruginosa from various clinical samples (e.g. urine, sputum, pus, body fluid, ET) received in central laboratory, department of Microbiology, GMC, Kota.

INCLUSION CRITERIA- Only isolates of *P. aeruginosa* from various clinical sample, patients above 18 years of age and male, female and transgender were included.

METHODS

Bacterial identification [5]

All isolated samples were sub-cultured on nutrient agar for pure isolation which were processed for appropriate phenotypic characterization based on morphology, culture and further tested by conventional biochemical tests including catalase test, oxidase test, OF test, lactose fermentation test, pigment production test and growth at 42°C leading to identification as *P. aeruginosa.*

Antibiotic susceptibility test-[6]

A routine antibiotic susceptibility test was performed for *P. aeruginosa* by Kirby-Bauer disk diffusion method. 0.5 McFarland standard was used to compare the inoculum turbidity. The inoculated plates were incubated overnight at 37 ^{0} C. Various antibiotics such as Amikacin-AK 30mcg, Gentamicin-GEN 10mcg, Piperacillin75mcg /tazobactam 10mcg - PIT, Ciprofloxacin-CIP 5mcg, Imipenem-IPM10 mcg, Cefepime-CPM 30mcg, Aztreonam-AT 30mcg, Ceftazidime-CAZ 30mcg and Meropenem-MRP 10mcg were used. The results were recorded by measuring the inhibition zone as sensitive(S), intermediate(I) and resistant(R), as per CLSI 2022 guidelines.[6] As a quality control, we were used *P. aeruginosa* ATCC 27853.

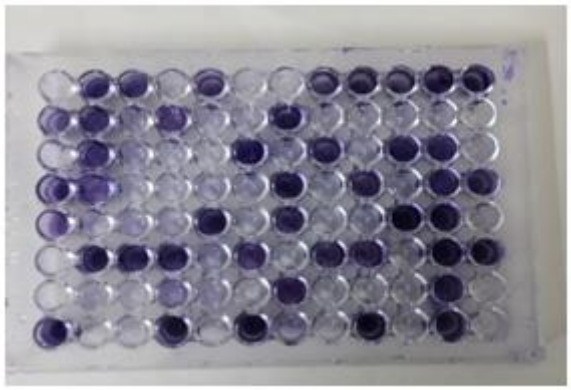
Phenotypic Method:

- **Microtiter Plate Assay** (Quantitative):[5,7]

1.Detection of Biofilm Production by tissue culture plate method-Organism isolated from fresh agar plates was inoculated in 10 mL TSB (Trypticase soya broth) with 1% glucose and incubated at 37^{0}C for 24 h. The culture was diluted 1:100 with fresh medium. 96 well flat bottom tissue culture plates were filled with 0.2 mL of diluted cultures individually. Uninoculated TSB was used as a control for sterility and non-specific binding of media. The plates were then incubated at 37^{0}C for 18-28h.After incubation, the plates were gently tapped for the removal of unbound content. The wells were then washed with 0.2mL of phosphate buffer saline (Ph7.4) four times. Biofilm formed by bacteria adherent to the wells was fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using microELISA auto reader at wavelength 490 nm. These OD values was considered as an index of bacteria adhering to surface and forming biofilm.

1. Mean OD =< 0.120 (Non\Weak Biofilm production)
2. Mean OD = 0.120-0.240 (Moderate biofilm production)
3. Mean OD=>0.240 (Strong Biofilm production)

Image 1-Biofilm formation by Microtiter plate method



1. **Detection of MBL Production:**

Screening and Confirmation of MBLs:[6]

The *Pseudomonas aeruginosa* isolates which were found to be resistant to Ceftazidime, Imipenem and Meropenem by Kirby – Bauer disc diffusion method was selected and the minimum inhibitory concentration of resistant isolates was determined and subjected to phenotypic detection methods such as Modified Hodge Test. ATCC 27853 *Pseudomonas aeruginosa* was used as a negative control.

Modified Hodge Test (MHT) [6,8]

- ATCC *E.coli* 25922 inoculum was prepared in 0.5 Mc Farland standards & lawn culture was made on Mueller-Hinton agar plate.
- Meropenem disc (10µg) was kept in the centre of the lawn.
- Colonies of Meropenem or Ceftazidime resistant isolates were taken & inoculated from edge of the disc to edge of the plate in a straight line & incubation was done at 37°Cover night.
- The formation of a clover leaf like indentation along the test strains indicated carbapenemase production.

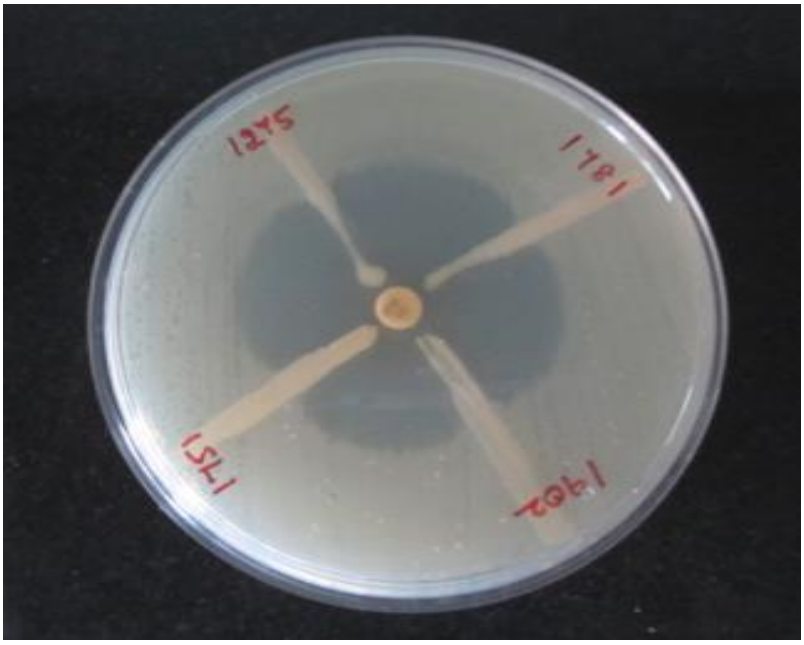


Image 2 - Modified Hodge Test - MBL Producer

RESULTS

During the study period from January 2023 to March 2025, a total of 225 isolates of *Pseudomonas aeruginosa* were collected from various clinical samples. Phenotypic identification of the isolates was performed using standard bacteriological methods, including Gram staining, colony morphology, and biochemical tests.

Among the 225 isolates, 50 (22.2%) were identified as MBL (Metallo-β-lactamase) producers, while 175 (77.8%) were non-MBL producers. (Table-2,Fig.-1) Out of the total isolates, 62 (27.6%) were biofilm producers and 163 (72.4%) were non-biofilm producers. Among the 62 biofilm-producing isolates, 41 (66.1%) were MBL producers and 9 (14.5%) were non-MBL producers.(Table-3.Fig-2)

A significant association was observed between biofilm formation and MBL production. Biofilm-producing isolates exhibited higher levels of antibiotic resistance compared to non-biofilm producers.

Table-1 Distribution of Biofilm-producer and non-MBL producers
(N=225)

Biofilm-producers (%)	Non-biofilm Producers (%)
62(27.6%)	163(72.4%)

Table :2 Distribution of MBL producer and non-MBL producers
(N=225)

MBL-producers	Non-MBL Producers
50(22.2%)	175(77.8%)

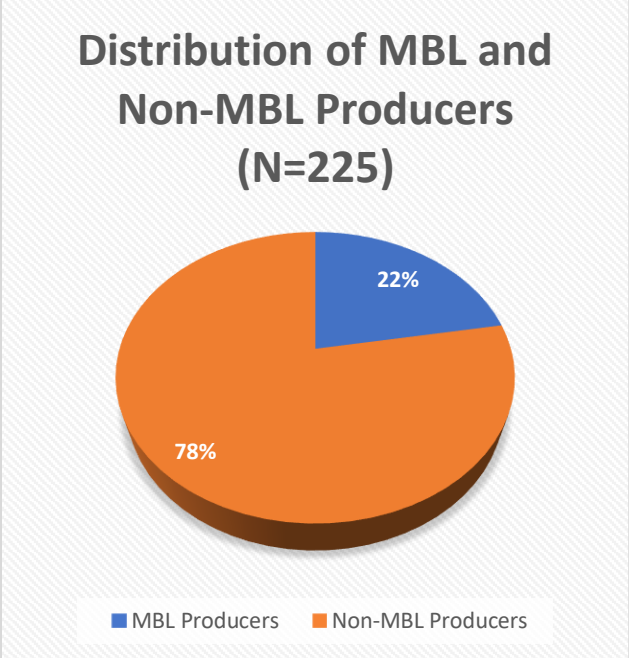


Figure-1 Distribution of MBL producer and non-MBL producers

Table 3: Correlation Between MBL Production and Biofilm Formation

Virulence factor	MBL Producers (%) (N=50)	Non-MBL Producers (%) (N= 175)
Biofilm Producers (n = 62)	41 (66.1%)	21 (33.9%)
Non-Biofilm Producers (n = 163)	9 (5.5%)	154 (94.5%)

P-value: *Significant* ($p < 0.001$)

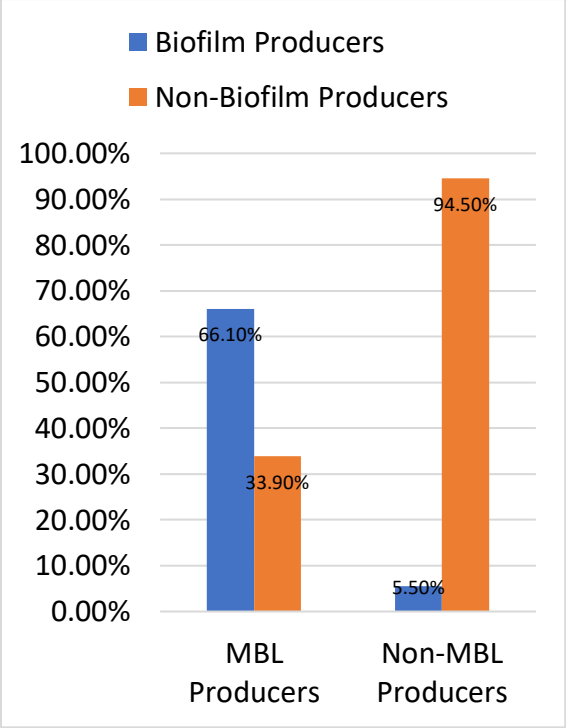


Figure 2: Correlation Between MBL Production and Biofilm Formation

DISCUSSION

Pseudomonas aeruginosa is an opportunistic pathogen known for its intrinsic resistance mechanisms and ability to acquire additional resistance genes, making treatment highly challenging. The production of Metallo-β-lactamases (MBLs) confers resistance to carbapenems, which are considered antibiotics of last resort. Additionally, biofilm formation enhances the pathogen's persistence and resistance to antimicrobial agents.[9]

In the present study, biofilm-forming ability was determined using the microtiter plate method. Among a total of 225 isolates of *P. aeruginosa*, 62 (27.6%) were identified as biofilm producers. This finding is comparable with the studies by Shrestha et al.[10] (33%) and Kulkarni DM et al.[11] (26.6%). However, it contrast with the studies done by Nepal et al.[12] and Costerton JW et al.[13], who reported higher biofilm production rates of 83.33% and 73.68% respectively. This variation may be attributed to differences in geographical location, the type of study, the nature of clinical specimens, and the method used for biofilm detection.

The disk diffusion test was used as a screening method for carbapenem resistance, following CLSI guidelines[6] (2022). In the present study, 35.8% of *P. aeruginosa* isolates were resistant to meropenem. Similar rates were reported by Manoharan et al.[14] (31.46%) and Adesola Olalekan et al.[15] (44%). However, other studies reported higher resistance rates, such as 56% by Sonarika et al.[16], 51% by Raghdaa A et al.[17], and 72.7% by Santosh Kumar Yadav et al.[18] These variations may be attributed not only to differences in sample size and clinical settings but also to factors such as the type and source of clinical specimens, variations in local antibiotic prescribing practices, infection control policies, regional antimicrobial resistance trends, and differences in laboratory methodologies, including the choice of carbapenem used for testing and interpretation criteria.

MBL production was detected using the Modified Hodge Test (MHT). In this study, out of 225 isolates of *P. aeruginosa*, 50 (22.2%) were MBL producers. Similar findings from Jayalakshmi et al.[19] (33%) and Vinita Choudhary et al.[20] (20%). In contrast, Shikha Ranjan et al.[21] and P. Samatha et al.[22] reported much higher MHT-positive rates of 87.5% and 86.44%, respectively. These discrepancies may be due to differences in sample size, types of clinical specimens, and regional epidemiology.

In the present study, out of the 62 biofilm-producing isolates, 41 (66.1%) were MBL producers and only 9 (14.5%) were non-MBL producers. This shows that the majority of biofilm producers were also MBL-positive. Conversely, among the 163 non-biofilm-producing isolates, only 21 (33.9%) were MBL producers, while a large majority, 154 (94.5%), were non-MBL producers. These findings indicate a strong positive correlation between MBL production and biofilm formation ($p < 0.001$), suggesting that strains capable of producing MBLs are more likely to also form biofilms. Similar study conducted by Khalil et al. (2020), Farzana et al. (2011) They reported that 63% and 60% of biofilm-forming *P. aeruginosa* were also MBL producers, aligning closely with the current study's finding of 66.1%.

Promodhani et al. (2019) observed that only 40% of biofilm producers were MBL positive, and a majority of non-biofilm producers were MBL-negative, indicating a weaker correlation compared to the presen findings.this may be due to Study Population Differences, Geographic and Temporal Variability, Different Detection Methods and Clonal Distribution.

CONCLUSION

The study found a positive correlation between MBL-producing *Pseudomonas aeruginosa* isolates and Biofilm formation, requiring early identification, infection control measures, and an all-inclusive antimicrobial therapy protocol to reduce their spread in medical settings. MBL detection remains contentious, but clinical laboratories need a quick method to identify resistant *Pseudomonas aeruginosa*.

Regular surveillance of resistance patterns and biofilm-forming capabilities of *P. aeruginosa* is crucial for guiding appropriate antimicrobial therapy. This study provides critical insights into the epidemiology of MBL production and biofilm formation in a tertiary care setting, underscoring the need for enhanced diagnostic and therapeutic approaches.

The strong correlation between biofilm formation and MBL production in P. aeruginosa highlights the urgent need for early detection and targeted therapy. Routine screening for MBL and biofilm production in clinical isolates should be implemented in tertiary care hospitals to improve infection control. Novel therapeutic approaches such as quorum sensing inhibitors, biofilm disruptors, and phage therapy need further research to combat these highly resistant infections.

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Conflicts of Interest: The authors declare no conflicts of interest.

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