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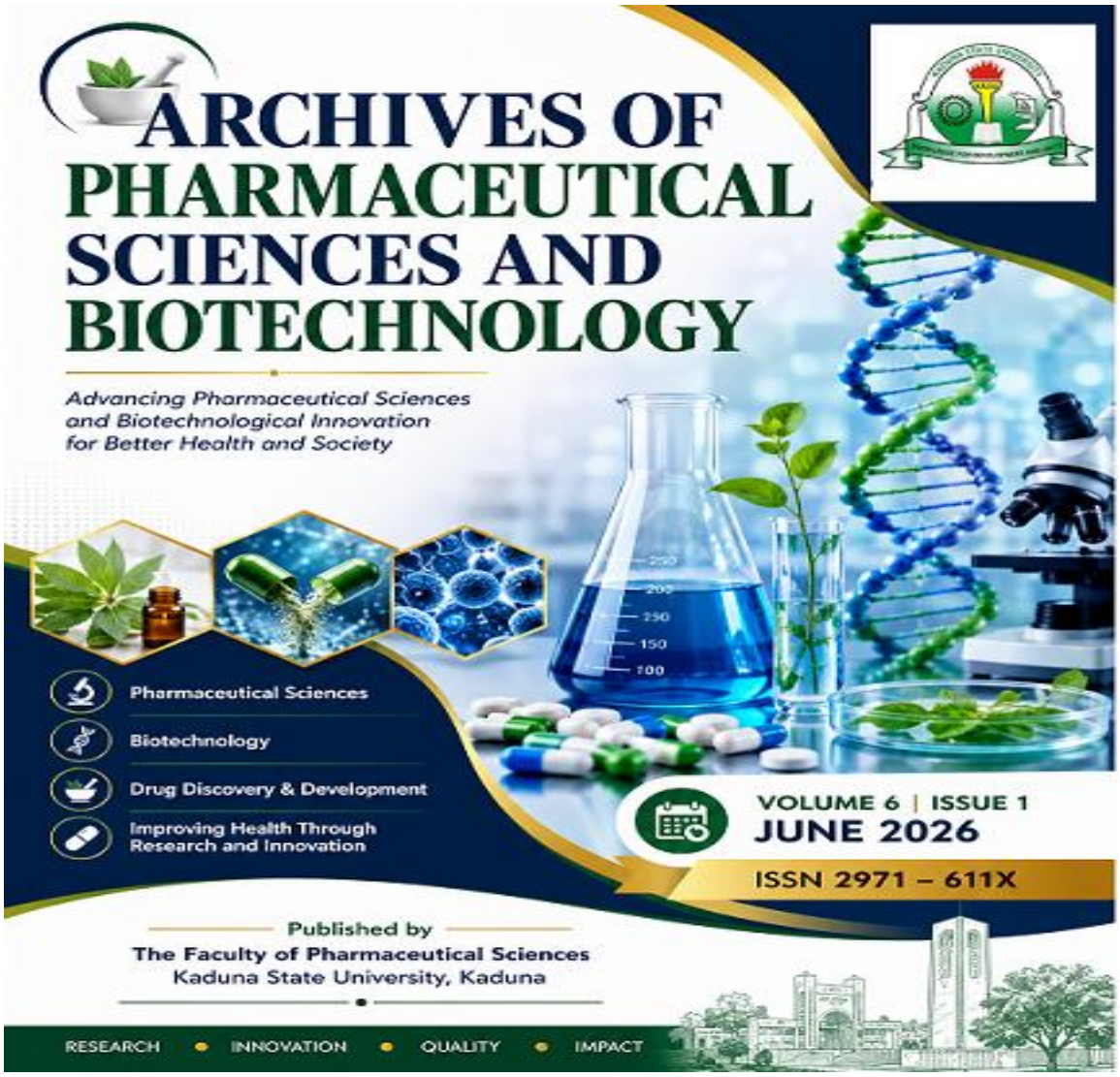

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MOLECULAR CHARACTERIZATION OF BIOFILM FORMATION IN MULTIDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA* ISOLATED FROM CLINICAL AND ENVIRONMENTAL SOURCES

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ABTSRACT

Pseudomonas aeruginosa is a major opportunistic pathogen implicated in persistent healthcare-associated infections with poor treatment outcomes. Its capacity to develop multidrug resistance (MDR) and to establish biofilms significantly complicates clinical management. This study investigated the biofilm-forming ability, antimicrobial resistance profile, and distribution of selected biofilm-associated genes among MDR *P. aeruginosa* isolates recovered from clinical and environmental sources. A total of 414 samples comprising clinical specimens (urine, wound swabs, ear swabs, high vaginal swabs, and catheter swabs) and environmental samples (soil, air, water, toilet seats, window sills, hospital beds, and door handles) were collected from three tertiary hospitals. Isolation and identification of *P. aeruginosa* were performed using standard microbiological methods in accordance with CLSI guidelines and confirmed with the VITEK 2 system. Antimicrobial susceptibility testing was conducted using the modified Kirby–Bauer disc diffusion method. Multidrug resistance was defined as resistance to at least one agent in three or more antimicrobial classes. Biofilm formation was quantified using the microtiter plate crystal violet assay, while polymerase chain reaction (PCR) was employed to detect biofilm-associated genes (algD, pslD, lasR, pelF, and ppgL). Out of 101 confirmed *P. aeruginosa* isolates, 43 (42.6%) were of clinical origin and 58 (57.4%) were environmental. Wound swabs yielded the highest number of clinical isolates, while soil samples accounted for the highest environmental recovery. Multidrug resistance was observed in 20 (19.8%) isolates, with a higher proportion among clinical sources. Biofilm production was detected in 31 (30.6%) isolates, comprising 5% strong, 11% moderate, and 14.9% weak biofilm producers. Among biofilm-producing isolates, 10 (32.3%) were MDR. Molecular analysis revealed the presence of algD (80%), pslD (60%), lasR (40%), and ppgL (30%), while pelF was not detected. A statistically significant association was observed between strong biofilm formation and multidrug resistance ($p < 0.05$). The findings highlight the clinical and environmental significance of biofilm-forming MDR *P. aeruginosa*. Targeted infection-control strategies and biofilm-directed therapeutic approaches are essential to mitigate the public health burden posed by this pathogen.

Keywords: *Pseudomonas aeruginosa*, multidrug resistance, biofilm, antimicrobial resistance, biofilm genes, clinical and environmental microbiology.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium recognized as one of the leading causes of healthcare-associated infections, particularly among immunocompromised individuals. It

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is frequently implicated in wound infections, urinary tract infections, respiratory tract infections, and device-associated infections, accounting for a substantial proportion of nosocomial disease burden worldwide (Wilson *et al*., 2023; Nikhil *et al*., 2023). The clinical importance of *P. aeruginosa* is largely attributable to its remarkable metabolic versatility, environmental persistence, and intrinsic resistance to multiple antimicrobial agents(Diggle and Whiteley, 2020; Itani *et al*., 2025).Antimicrobial resistance in *P. aeruginosa* arises from a combination of intrinsic mechanisms, including low outer-membrane permeability and efflux pump activity, as well as acquired resistance determinants mediated by mobile genetic elements (Zheng *et al*.,2019; Giovagnorio*et al*.,2023). The global emergence of multidrug-resistant (MDR) strains has severely limited therapeutic options and has been associated with increased morbidity, mortality, and healthcare costs (Wang, 2025). Beyond antimicrobial resistance, the ability of *P. aeruginosa* to form biofilms represents a critical virulence factor (Tuon *et al*., 2022). Biofilms are structured microbial communities embedded within a self-produced extracellular polymeric matrix that enhances bacterial survival under hostile conditions. Biofilm-associated *P. aeruginosa* exhibits markedly increased tolerance to antibiotics and host immune responses, facilitating persistent and recurrent infections (Mirghani *et al*., 2022; Sharma *et al*., 2023). Biofilms also promote horizontal gene transfer, thereby accelerating the dissemination of resistance genes within microbial populations (Michaelis and Grohmann 2023; Pallee *et al*., 2023; Almatroudi, 2025). Biofilm formation in *P. aeruginosa* is regulated by complex genetic networks involving quorum sensing and the coordinated expression of polysaccharide biosynthesis genes such as *algD*, *pslD*, and *pelF*, as well as regulatory genes including *lasR* and *ppgL* (Vetrivel *et al*., 2021; Rajabi *et al*., 2022; Tuon *et al*., 2022). Although several studies have reported MDR and biofilm-forming *P. aeruginosa* globally, data on the molecular determinants of biofilm formation among clinical and environmental isolates in North-Central Nigeria remain limited. This study therefore aimed to evaluate antimicrobial resistance patterns, biofilm-forming capacity, and the distribution of selected biofilm-associated genes among *P. aeruginosa* isolates from clinical and environmental sources.

MATERIALS AND METHODS

Study Design and Study Area

This cross-sectional study was conducted over a seven-month period (December 2021 to June 2022) in three hospitals: Benue State University Teaching Hospital (BSUTH), Federal Medical Centre (FMC), and General Hospital (GH), Makurdi, Nigeria. Clinical isolates and environmental samples were collected and analyzed for the presence of *P. aeruginosa*.

Sample Size Determination

Sample size was calculated using the Thrusfield (2005) formula, based on previously reported prevalence rates of 12% % by Omoni, *et al.*, (2015) for clinical samples and 20% for environmental samples by Gad *et al*., (2017), with a 95% confidence interval and 5% margin of error. A total of 162 clinical specimens and 252 environmental samples were collected.

Collection and Identification of Isolates

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Clinical specimens were obtained from routine diagnostic submissions to hospital microbiology laboratories, while environmental samples were collected from selected hospital surroundings and surfaces. Samples were cultured on cetrimide agar and other appropriate media. Presumptive *P. aeruginosa* isolates were identified using standard biochemical tests in line with CLSI guidelines and further confirmed using the VITEK 2 automated system. Confirmed isolates were stored at −4 °C until analysis.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the modified Kirby–Bauer disc diffusion method on Mueller–Hinton agar. Inocula were standardized to 0.5 McFarland turbidity. Zone diameters were measured and interpreted according to CLSI (2020) criteria. Isolates resistant to at least one agent in three or more antimicrobial classes were classified as MDR.

Quantitative Biofilm Assay

Biofilm formation was assessed using the microtiter plate crystal violet method. Isolates were cultured in brain heart infusion broth supplemented with 2% glucose and incubated in 96-well plates. After incubation, wells were washed, stained with crystal violet, and the bound dye solubilized. Optical density was measured at 545 nm. Biofilm production was categorized as strong, moderate, or weak based on optical density values relative to controls.

Molecular Detection of Biofilm-Associated Genes

Genomic DNA was extracted from MDR biofilm-producing isolates using a commercial DNA extraction kit. PCR amplification was performed for *algD*, *pslD*, *pelF*, *lasR*, and *ppgL* genes using specific primers. PCR products were resolved by agarose gel electrophoresis.

Table 1: Primers for Biofilm Associated genes

Primer name	Sequence 5'-3'	Amplicon size (bp)	Annealing temp(°c)	References
algD	F-CTACATCGAGACCGTCTGCC R-GCATCAACGAACCGAGCATC	593	45	Banar *et al*., 2016
Pelf	F-GAGGTCAGCTACATCCGTCG R-TCATGCAATCTCCGTGGCTT	789	45	Banar *et al*., 2016
pslD	F-TGTACACCGTGCTCAACGAC R-CTTCCGGCCCGATCTTCATC	369	45	Banar *et al*., 2016
Ppgl	F-GTGGTGGGGACCTATACCGAA R- GTAGTTGGCGACGAACAGGTA	327	45	Rajabi, *et al*.,2022
LasR	F-AAGGAAAGTGTTGCAGTGGTC R-GAGCAGTTGCAGATAACCGA	720	45	Rajabi,*et al*.,2022

Ethical Approval

Ethical approval for this study was obtained from the ethics and research committees of the selected hospitals. The clearance numbers granted were BUSTH/ADM/TRA/279//1/52 for Benue State University Teaching Hospital (BSUTH), FMH/FMC/MED.108/VOL.I/X for Federal Medical Centre (FMC), and GH/ADM/TRA/123/1/30 for General Hospital (GH).

Statistical Analysis

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Data were analyzed in IBM SPSS v20 using descriptive statistics, with results presented as frequencies, percentages, tables, and graphs.

RESULTS

Over a seven-month period, 101 *Pseudomonas aeruginosa* isolates were recovered from the 414 samples analyzed, giving an overall prevalence of 24.4%. Of these, 58 (57.4%) were obtained from environmental sources, while 43 (42.6%) were recovered from clinical samples from Benue State University Teaching Hospital (BSUTH), Federal Medical Centre (FMC), and General Hospital (GH), Makurdi. Among clinical specimens, wound samples yielded the highest number of isolates (15; 14.9%), whereas soil samples accounted for the highest recovery among environmental sources (13; 12.9%) (Table 2). The distribution varied across hospitals: BSUTH contributed 46.5% of clinical and 36.2% of environmental isolates, FMC accounted for 46.5% clinical and 29.3% environmental isolates, while GH yielded fewer clinical isolates (7%) but a higher proportion of environmental isolates (34.5%).'

Table 2: Distribution of *Pseudomonas aeruginosa* Isolated from Various Clinical and Environment Sources

Sources Sample	Hospitals			Total	%
	Gen. Hospital	BSUTH	FMC		
Clinical Samples					
Wound Swab	2	7	6	15	14.9
Ear Swab	0	3	5	8	7.9
Urine	0	6	4	10	9.9
High Vagina Swab	1	2	2	5	5
Catheter swab	0	2	3	5	5
Total (%)	**3(7)**	**20(46.5)**	**20(46.5)**	**43(100)**	**43(42.6)**
Environmental Samples					
Soil	5	4	4	13	12.9
Air	3	2	1	6	5.9
Toilet seats	3	3	4	10	9.9
Water	2	3	2	7	6.9
Window sills	3	4	2	9	8.9
Hospital Bed	2	2	3	7	6.9
Door Handles	2	3	1	6	5.9
Total (%)	**20(34.5)**	**21(36.2)**	**17(29.3)**	**58(100)**	**58(57.4)**
Sum Total	**23(22.8)**	**41(40.6)**	**37(36.6)**	**101(100)**	

Footnote: *Values in parentheses represent the percentage of isolates relative to the total number of isolates recovered (n = 101).*

Antibiotic susceptibility testing showed varying resistance patterns among the isolates (Table 3). Clinical isolates exhibited the highest resistance to colistin, with notable resistance to β-lactams, including ceftazidime (30.3%), cefixime (25.6%), and piperacillin (14%), as well as carbapenems such as imipenem (27.5%) and meropenem (16.3%). Environmental isolates showed the highest resistance to ceftazidime (53.4%),

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followed by piperacillin (24.1%), cefixime (10.3%), and meropenem (10.3%), while resistance to imipenem was low (1.7%). Resistance to aminoglycosides and fluoroquinolones was moderate and comparable across both sources. Overall, clinical isolates demonstrated higher resistance levels.

Table 3: Antibiotics Resistance of *Pseudomonas aeruginosa* Isolates From Clinical and Environmental Sources

S/no	Antibiotics	%resistance of Clinical isolates	% resistance of Environmental Isolates
1	Ceftazidime	30.3	53.4
2	Cefexime	25.6	10.3
3	Piperacillin/tazobactam	14	24.1
4	Imipenem	27.5	1.7
5	Meropenem	16.3	10.3
6	Amikacin	11.6	13.8
7	Gentamicin	16.6	13.8
8	Levofloxacin	9.3	17.2
9	Ciprofloxacin	23.2	6.9
10	Colistin	44.2	15.2

Footnote: *Values in parentheses represent the percentage of resistant isolates from clinical and environmental source relative to the total number of isolates recovered (n = 101).*

Multidrug resistance was detected in 19.8% (20) of the isolates, while non-MDR were 80.2% (81) (Figure 1).

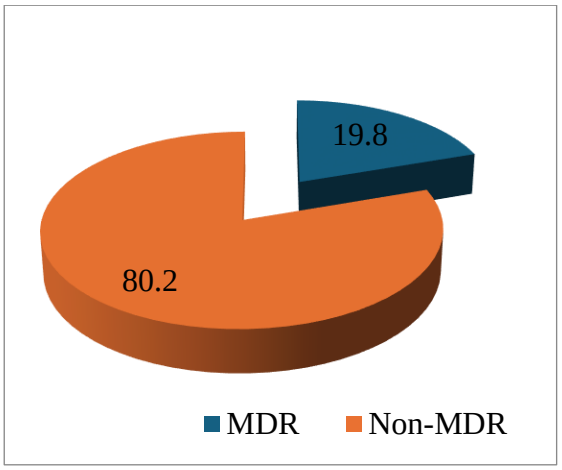

Figure 1: Distribution of Multidrug-resistant *Pseudomonas aeruginosa* isolates from Clinical and Environmental Sample source (n=101)

Analysis of the MDR isolates showed that 12(60%) were from the clinical isolates, while those of environmental isolates were 8(40%) **(**Table 4).

Table 4: Distribution of Multidrug-Resistant (MDR) Phenotypes among Clinical and Environmental *Pseudomonas aeruginosa* Isolates (n=24)

Source	Total isolates	MDR isolates
Clinical	58	12
Environment	43	8
Total	**101**	**20**

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Quantitative assessment of Biofilm formation showed 5% of isolates as strong producers, while11% moderate and 14.9% weak producers (Figure 2).

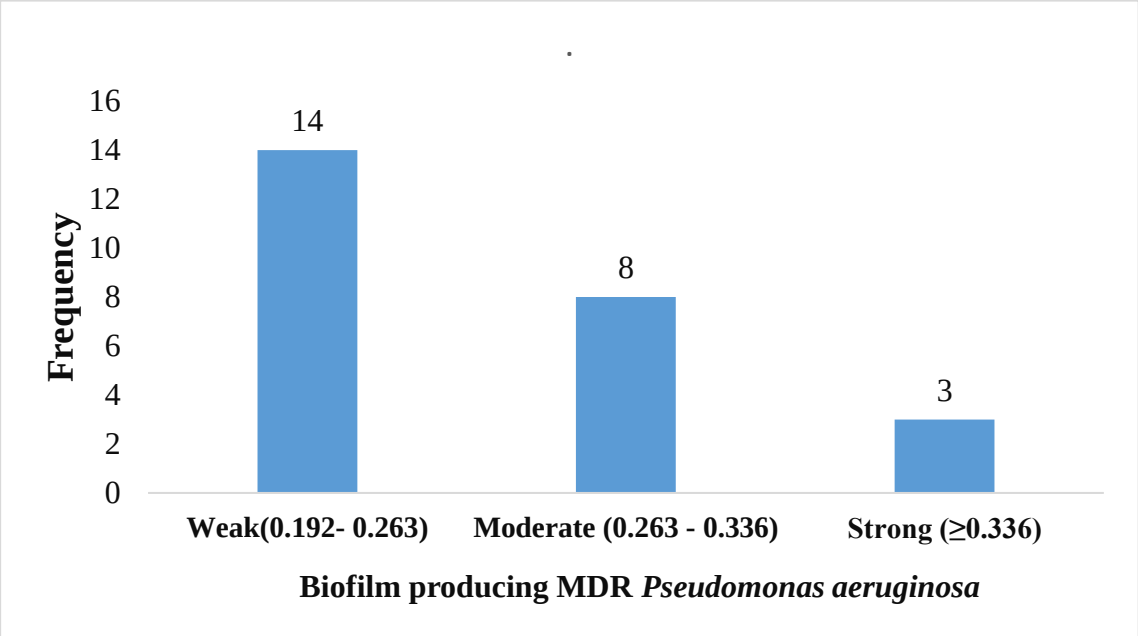

Figure 2: Quantification of Biofilm production in MDR *Pseudomonas aeruginosa* isolates (n = 101)

Majority of the MDR- biofilm-producing *Pseudomonas aeruginosa* isolates were from clinical source 7(70%) while 3(30%) were from environmental sources as shown in Table 4.

Table 4: Distribution of Multidrug Resistance, Biofilm producing *Pseudomonas aeruginosa* Isolates from Clinical and Environmental sources

Source	Total isolates	MDR isolates	Biofilm producers	MDR + Biofilm producers
Clinical	58	12	19	7
Environment	43	8	12	3
Total	101	20	31	10

Of the MDR-Biofilm producing *Pseudomonas aeruginosa*, the higher occurrence of *the isolates* were in Clinical isolates than environmental isolates, with most recovered isolates from wound samples, followed by HVS, urine, and ear. Environmental isolates were obtained from door handles, toilet seats, and hospital beds, presented in Figure 3.

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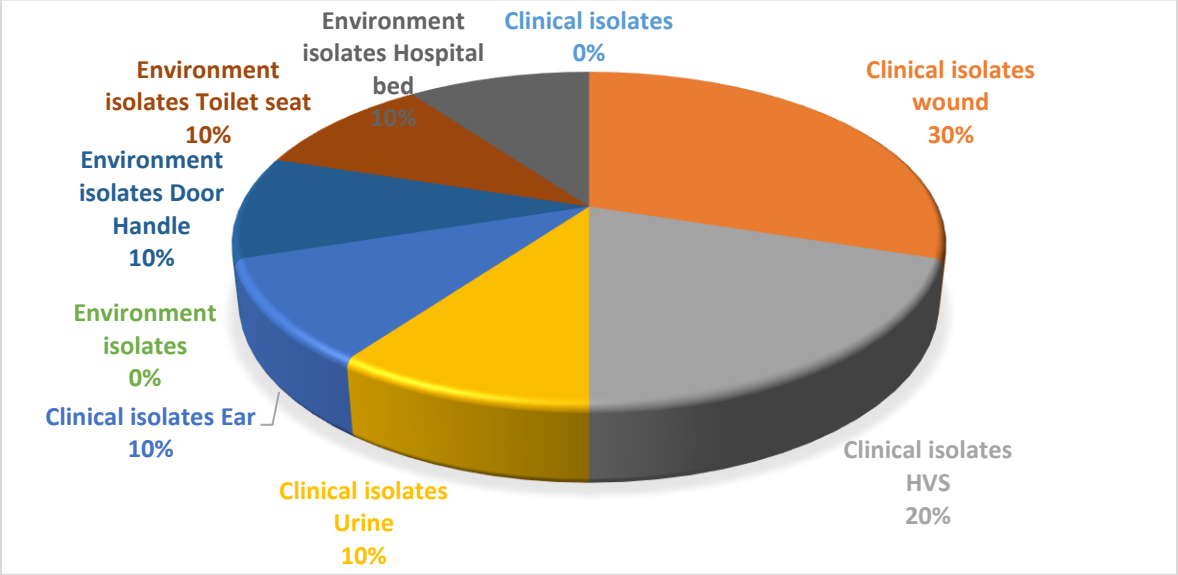

Figure 3: Source distribution of MDR, Biofilm-Producing *Pseudomonas aeruginosa* Isolates from clinical and environmental isolates

Amplification of biofilm associated genes in MDR biofilm-producing *Pseudomonas aeruginosa* isolates showed that *pelF* was absent in all isolates, while *algD* was present in 80% of isolates across both clinical and environment sources (Plate1). The *pslD* gene was detected in 60% of isolates, predominantly in clinical isolates (50%) and few in environmental isolates (10%) (Plate 1).

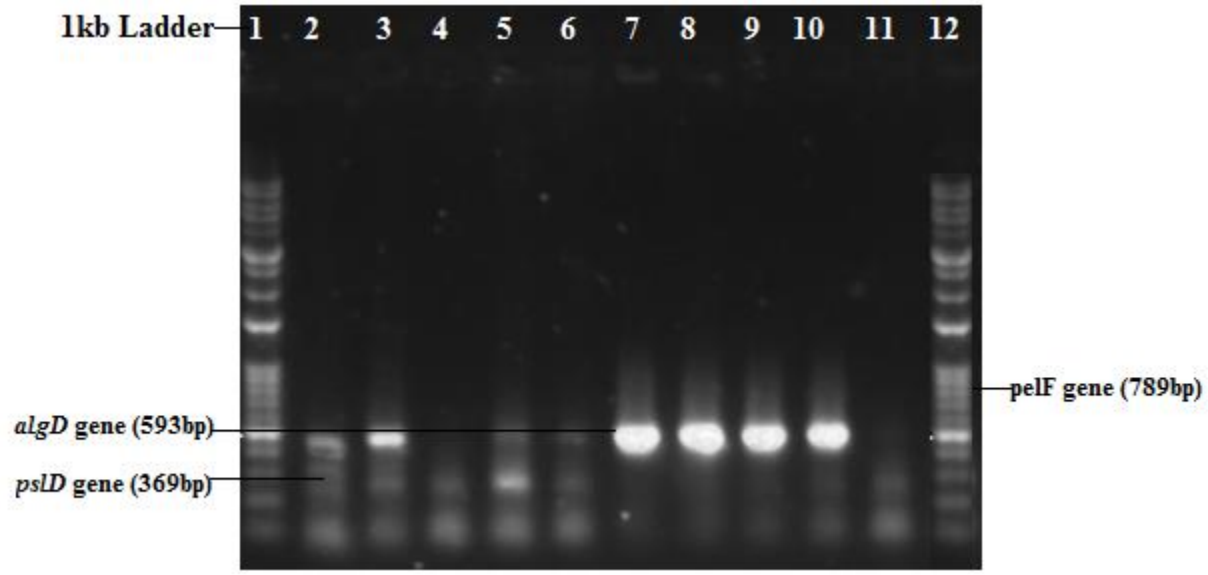

Plate 1: Agarose gel electropherogram of PCR Lane 2-11: Amplification of Biofilm genes of *pelF* (789bp), *algD* (593bp) and *pslD* (369bp).

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Lane 1(1Kb ladder), Lane2 (WS4), Lane3 (WS25), Lane4 (WS5), Lane5 (ES19), Lane6 (HVS3), Lane7 (U9), Lane8 (HVS23), Lane9 (SS5), Lane10 (TSS11), Lane11 (DHS15), Lane12 (1Kb ladder)

While *lasR* (720 bp) as detected in 40% of isolates, mainly from clinical sources (30%) and 10% from environmental source. Furthermore, the *Ppgl* gene (327 bp) was detected in 30% of isolates, with 20% from clinical and 10% from environmental samples.

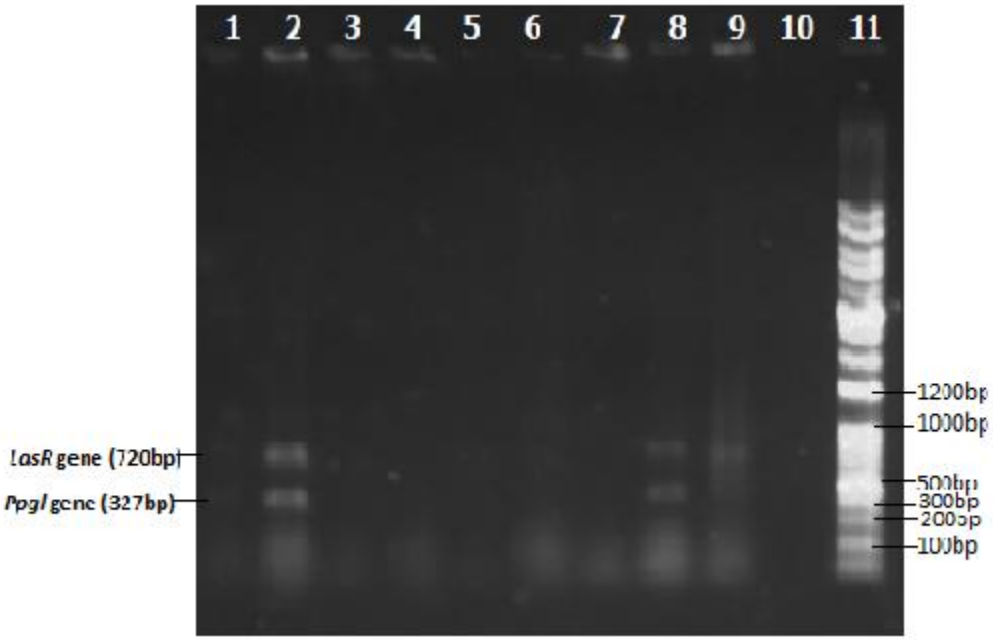

Plate 2: Agarose gel electropherogram of PCR Lane 1-15- Amplification of biofilm genes of Ppgl (bp) and LasR (bp)
Lane1 (WS4), Lane 2 (WS25), Lane 3 (WS5), Lane 4 (ES19). Lane 5 (HVS3), Lane 6 (U9), Lane 7(HVS23), Lane 8 (SS5), Lane 9 (TSS11), Lane 10 (DHS15), Lane11 (1Kb ladder).

Distribution of the amplified biofilm associated genes in MDR biofilm-producing *Pseudomonas aeruginosa* isolates showed (Figure 4).

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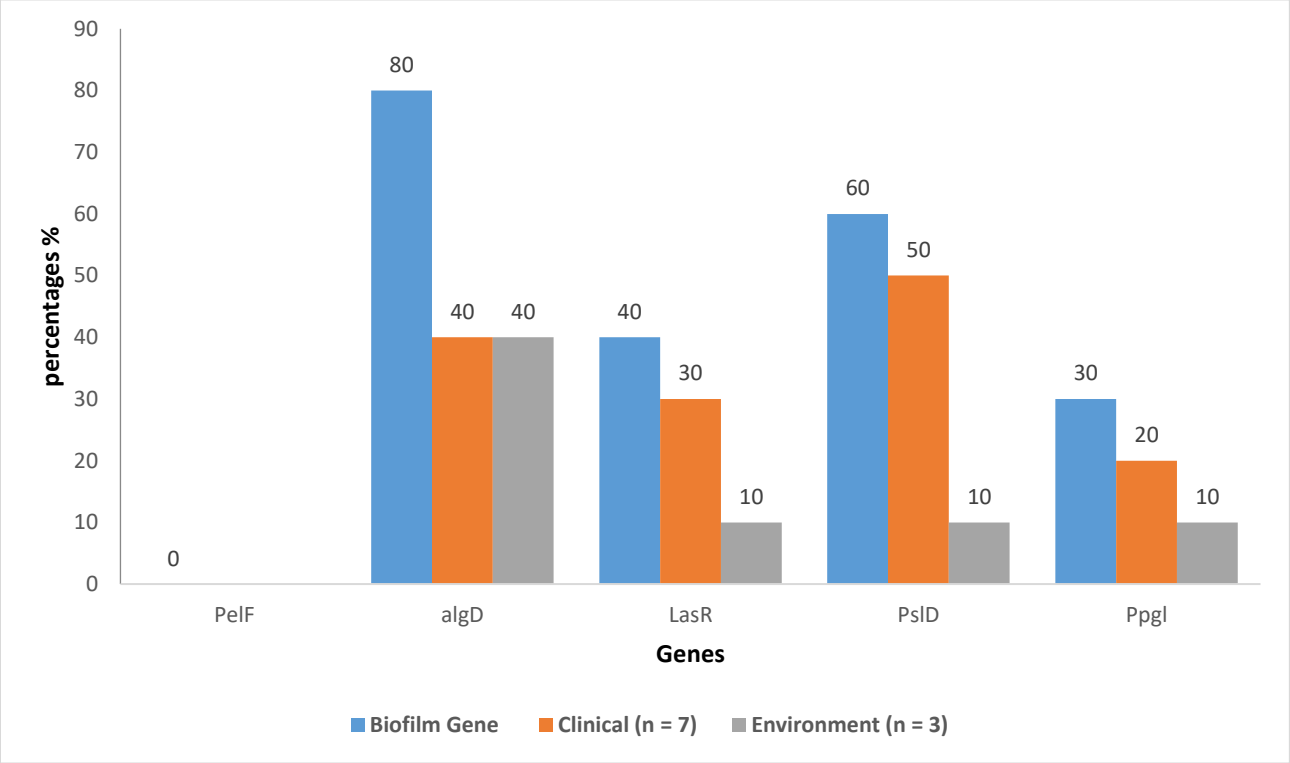

Figure 4: Distribution of Biofilm genes detected in Multidrug resistant *Pseudomonas aeruginosa* isolates (n =10)

DISCUSSION

The rise of multidrug-resistant *Pseudomonas aeruginosa* in healthcare settings limits treatment options, highlighting the need for ongoing research to understand and address this burden (Nihil *et al*., 2023; Schwartz *et al*., 2024). Finding from this study reveal high prevalence and wide distribution of *Pseudomonas aeruginosa*, across the clinical and environmental sources from Benue State University Teaching Hospital (BSUTH), Federal Medical Centre (FMC), and General Hospital Makurdi. There was slightly higher prevalence of *Pseudomonas aeruginosa*, isolates in the environment (57.4%) compared to clinical samples (42.6%), emphasizing its epidemiological importance in both the environment and human infections (Reynolds *et al*., 2021). Wounds and soil had the highest proportions from clinical and environmental sources respectively. The higher recovery from wounds highlights the role of *Pseudomonas aeruginosa* as an opportunistic pathogen commonly associated patients with weak immune system (Klrissa *et al*., 2016; Johan *et al*., 2017), while its presence in soil demonstrates its adaptability, widespread distribution and ability to survive under nutrient-limited conditions (Zheng *et al*., 2019). This finding aligns with report of Ezeador *et al*., (2020), with a similar prevalence of *Pseudomonas aeruginosa* 18.3% (22) and 86.4% *Pseudomonas aeruginosa* isolates from the environment and 13.6% from clinical source. The study conducted in northern Nigeria by Adejobi *et al*., (2023) reported higher prevalence of

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66.7% from the environment notwithstanding Abdurrahman *et al*., (2025) comparatively lower prevalence report of 12.8% from clinical samples. Hospital distribution showed BSUTH with the highest recovery rate, followed by FMC and the General Hospital. Variation in the distribution of *Pseudomonas aeruginosa* can be due to differences in patient turnover, contaminations levels and genetic adaptation (Finnan *et al*., 2004; Catherine *et al*., 2021; Kiyaga *et al*., 2022), and the occurrence of *Pseudomonas aeruginosa* in both clinical and environmental in same hospital indicates possible cross-transmission within hospital settings and possible outbreak risk (Ruiz *et al*., 2025). This study showed that the clinical *Pseudomonas aeruginosa* isolates exhibited higher resistance to most antibiotics compared to environmental isolates. The Clinical isolates showed higher resistance to polymyxin, β-lactam, aminoglycosides and fluoroquinolones antibiotics, while environmental isolates were more resistant to imipenem, ticarcillin, cefixime, carbapenem and β-lactam antibiotics. The observed high resistant to colistin, a polymyxin was inconsistent to low resistance report by Salem *et al*., 2024. The higher resistance in clinical isolates is likely due to frequent antibiotic exposure and selective pressure in hospital settings (Shivangee *et al*., 2024). These findings agree with Adeyemo *et al*. (2024) in Osogbo, who also reported higher resistance among clinical isolates. These differences in resistance levels across the sources might be due to geographic differences in antimicrobial prescriptions, infection control practices and spread of resistance gene (Salam *et al*., 2023; Sirwan *et al*., 2024), contributing to multidrug resistance (MDR) strains (Magiorakos *et al*., 2012; Salam *et al*., 2023; Oyedeji, 2025), a major public health menace. In this study, the MDR isolates were 20(19.8%) and slightly higher among clinical isolates. Comparable studies reported MDR rates ranging from 13.6% to 100%, including a lesser value of 13.6% from southeast Nigeria reported by (Ezeador, 2020), a higher rate of 51.7% in Iran (Pezhman *et al*. 2019), 72% in Osogbo (Adeyemo *et al*. 2024), 43% in Ghana (Odoi *et al*. 2021), 71.4% in Brazil (de Almeida Silva *et al*,. 2017), and 100% in Northern Nigeria (Adejobi *et al*,. 2021). The prevalence of multidrug-resistant *Pseudomonas aeruginosa* represents a significant public health concern because of its limited therapeutic options, high pathogenic potential, and capacity to persist in both clinical and environmental environments (Elfadadny *et al*., 2024; WHO, 2024).

In this study also, biofilm production was observed in 31 (30.6%) of the *Pseudomonas aeruginosa* isolates and this aligns closely with the 32% biofilm formation reported by Kulkarni *et al*. (2020), but contrasts with the higher rate of 84% reported by Salem *et al*., (2025). Notably, the majority of biofilm-producing isolates in this study originated from clinical sources, a pattern consistent with the observations of Salem *et al*., (2025). Further analysis of biofilm producers, shows 5% produced strong biofilms, 11% moderate and 14.9% weak producers. This distribution aligns with report by Salem *et al.*, (2025), who reported moderate to strong biofilm forming ability. This is consistent with report by Swamy *et al.* (2024) in India who observed that all *Pseudomonas aeruginosa* isolates from clinical sources produced biofilms, with 56% strong, 34% moderate, and 10% weak producers. Report by Salem *et al*., (2024) of

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moderate to strong biofilm forming ability agrees with these findings. Similarly, Karami *et al*. (2019), reported biofilm formation in 94.8% of clinical and 80% of environmental isolates. The biofilm-associated genes in MDR biofilm-producing *Pseudomonas aeruginosa* isolates showed that the pelF gene was absent in all the isolates, possibly due to genetic variation, while *algD* was present in 80% of isolates in both clinical and environmental isolates. The *pslD* gene was detected in 60% of isolates, predominantly in clinical (50%) compared to environmental (10%) samples, indicating a stronger clinical association shown in Plate 1. While *lasR* (720 bp) as detected in 40% of isolates, mainly from clinical sources (30%), suggesting stronger clinical relevance. The *Ppgl* gene (327 bp) was detected in 30% of isolates, with 20% from clinical and 10% from environmental samples, indicating moderate distribution across both sources shown in Plate 2. The significant association observed between strong biofilm formation and multidrug resistance, indicates biofilm formation enhances bacterial resistance to antibiotics and host defenses. The distribution of biofilm-associated genes observed in this study is comparable to the findings of Rajabi *et al*., (2022), who reported gene prevalence as follows: algD (78.6%), pelF (70.5%), pslD (36.6%), and Ppgl (0%). Detection of biofilm-associated genes in 50% of MDR *Pseudomonas aeruginosa* isolates from both clinical and environmental sources indicates a strong association between multidrug resistance and biofilm formation. This finding aligns with studies by Qian *et al*., (2022) that MDR isolates exhibited enhanced biofilm-forming ability. Similarly, Shah *et al.* (2024) in Dhaka, Bangladesh, found that 93.82% of hospital wastewater isolates were multidrug-resistant, with 71 of 76 MDR isolates forming biofilms. Consistently, Collins *et al*. (2022) in Nigeria observed a high biofilm formation rate of 57.4% among isolates, reinforcing the global association between multidrug resistance and biofilm production in *Pseudomonas aeruginosa.*

CONCLUSION

This study highlights the widespread occurrence of multidrug-resistant, biofilm-producing *Pseudomonas aeruginosa* in clinical and hospital environmental settings. The strong association between biofilm formation and antimicrobial resistance underscores the need for improved infection control, environmental hygiene, and biofilm-targeted therapeutic strategies to reduce persistence, transmission, and treatment failure.

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