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Characterization of biofilm formation and multi-drug resistance among *Pseudomonas aeruginosa* isolated from hospital wastewater in Dhaka, Bangladesh

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ABSTRACT

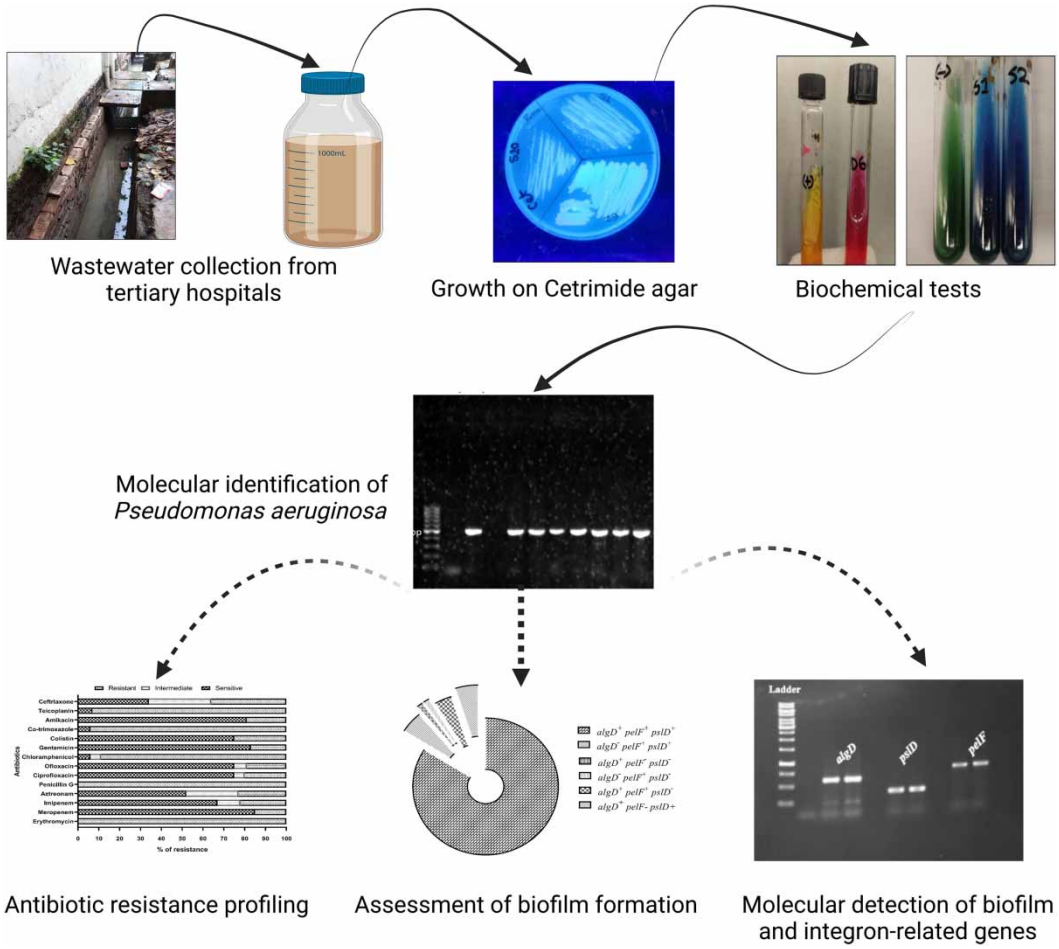
Hospital wastewater has been identified as a hotspot for the emergence and transmission of multidrug-resistant (MDR) pathogens that present a serious threat to public health. Therefore, we investigated the current status of antibiotic resistance as well as the phenotypic and genotypic basis of biofilm formation in *Pseudomonas aeruginosa* from hospital wastewater in Dhaka, Bangladesh. The disc diffusion method and the crystal violet assay were performed to characterize antimicrobial resistance and biofilm formation, respectively. Biofilm and integron-associated genes were amplified by the polymerase chain reaction. Isolates exhibited varying degrees of resistance to different antibiotics, in which >80% of isolates showed sensitivity to meropenem, amikacin, and gentamicin. The results indicated that 93.82% of isolates were MDR and 71 out of 76 MDR isolates showed biofilm formation activities. We observed the high prevalence of biofilm-related genes, in which *algD*$^+$*pelF*$^+$*pslD*$^+$ (82.7%) was found to be the prevalent biofilm genotypic pattern. Sixteen isolates (19.75%) possessed class 1 integron (*int1*) genes. However, statistical analysis revealed no significant association between biofilm formation and multidrug resistance ($\chi^2 = 0.35$, $P = 0.55$). Taken together, hospital wastewater in Dhaka city may act as a reservoir for MDR and biofilm-forming *P. aeruginosa*, and therefore, the adequate treatment of wastewater is recommended to reduce the occurrence of outbreaks.

Key words: antimicrobial resistance, Bangladesh, biofilm, hospital wastewater, *P. aeruginosa*, transmission

HIGHLIGHTS

- A high occurrence of *P. aeruginosa* was observed in hospital effluents.
- 93.82% of isolates were multidrug-resistant.
- Most of the isolates showed biofilm formation activities.
- 19.75% of isolates harbored class 1 integron genes.
- Hospital wastewater in Dhaka acts as a reservoir of MDR *P. aeruginosa*.

GRAPHICAL ABSTRACT



INTRODUCTION

Pseudomonas aeruginosa is a versatile opportunistic pathogen that causes a wide array of life-threatening infections, especially in immunocompromised individuals, leading to high morbidity and mortality (Kamali *et al.* 2020). It counters a wide variety of antibiotic attacks by employing intrinsic, acquired, and adaptive mechanisms of resistance. Intrinsic resistance is mediated by changes in the permeability of the outer membrane, efflux pumps, and the production of antibiotic-inactivating enzymes (Pang *et al.* 2019). Horizontal gene transfer (HGT) and mutation facilitate acquired resistance (Breidenstein *et al.* 2011). In addition, biofilm formation acts as a barrier that limits antibiotics' access to cells and thus promotes adaptive resistance (Drenkard 2003). Therefore, the increasing global prevalence and transmission of multidrug-resistant (MDR) *P. aeruginosa* poses a major challenge in the management of complicated infections (Moreira *et al.* 2013; Mirzaei *et al.* 2020). In 2017, the World Health Organization (WHO) listed Carbapenem-resistant *P. aeruginosa* as a critical priority pathogen to accelerate the development of new therapeutics (Tacconelli *et al.* 2017).

Pseudomonas isolates exhibit incredible adaptability and ability to survive in a variety of environments, such as soil, water, urban waste, hospital environments, and medical waste (Palleroni 1984). Earlier studies have identified hospital effluent as a breeding ground for the emergence and transmission of antimicrobial-resistant (AMR) bacteria in the environment (Miranda *et al.* 2015; Zhang *et al.* 2020; Wu *et al.* 2022). High amounts of antibiotics are used in hospitals, and most of them are not metabolized after ingestion; instead, a significant amount of antibiotics are released into the environment via human excretion (Yang *et al.* 2009). Those unmetabolized antibiotics exert selective pressure and, thus, induce the evolution of resistance (Skandalis *et al.* 2021). In addition, HGT, in particular conjugation, allows the spread of AMR-associated genes from pathogens to environmental microbes in both terrestrial and aquatic habitats, which can be further enriched through

anthropogenic activities (Von Wintersdorff *et al.* 2016; Bello-López *et al.* 2019). Here, integrons play an essential role with the class 1 integron-integrase gene being the most crucial (Zheng *et al.* 2020). The dissemination of MDR *P. aeruginosa* from hospitals to the natural environment may contribute to an increase in the number of community-acquired infections that have become a cause of concern for public health professionals (Slekovec *et al.* 2012). Previous studies described two outbreaks of MDR *P. aeruginosa* that were potentially associated with hospital waste systems (Breathnach *et al.* 2012).

Biofilm is one of the most prominent virulence factors of *P. aeruginosa*. It is usually made up of at least three different exopolysaccharides, including alginate, *Psl*, and *Pel* (Franklin *et al.* 2011). Alginate provides structural stability and protection to biofilm (Li *et al.* 2019; Kamali *et al.* 2020). The other two exopolysaccharides, *pel* and *psl*, are known to function as structural scaffolds that are required to maintain the biofilm's integrity (Colvin *et al.* 2012). Biofilm has a considerable role in AMR and chronic infections (Kunwar *et al.* 2021). About 65–80% of pathogenic infections in healthcare are associated with biofilm formation (Jamal *et al.* 2018). According to some studies, biofilm-forming *P. aeruginosa* strains can withstand ceftazidime, ciprofloxacin, and tobramycin at concentrations higher than those required to eradicate planktonic bacteria, signifying the protective role of biofilm in bacterial survival at stressed conditions (Anwar & Costerton 1990; Moriarty *et al.* 2007). The notorious persistence of *P. aeruginosa* in hospital settings is also favored by its antibiotic-resistant biofilm (Thi *et al.* 2020).

Infections caused by AMR bacteria have often been linked to the unsafe disposal of waste from healthcare facilities (Hocquet *et al.* 2016). These infections are common in areas where the prophylactic use of antibiotics is frequent, such as Bangladesh. This is primarily due to a lack of proper surveillance, low awareness regarding antibiotic consumption, and the presence of untrained healthcare professionals (Ahmed *et al.* 2019). The liquid medical waste is directly discharged into the municipal sewage system that pollutes the surrounding aquatic environments (Adnan *et al.* 2013). Bangladesh only processes 17% of its total wastewater. Hence, untreated waste may increase the dangers of infections by drug-resistant microbes. Even treated hospital wastewater has been linked to the spread of MDR microorganisms (Behnam *et al.* 2020). Several studies described the abundance of antibiotic-resistant bacteria, the mechanism of resistance, and the emergence of MDR strains in hospital liquid waste from different regions of Bangladesh (Islam *et al.* 2017; Rabbani *et al.* 2017; Akther *et al.* 2018; Khan *et al.* 2022). However, adequate information is not available on the biofilm formation capacity of *P. aeruginosa* prevalent in hospital wastewater and its association with multidrug resistance. Therefore, we evaluated the multidrug resistance, biofilm formation, and their connection in *P. aeruginosa* from hospital effluents in Dhaka, Bangladesh.

MATERIALS AND METHODS

Sample collection

Untreated hospital wastewater samples ($N = 12$) were collected from four different hospitals in Dhaka city, Bangladesh between June and September 2021 (see Supplementary material). About 500 mL of wastewater was collected from three different discharge points in each hospital. Following collections, samples were immediately transported to the laboratory for microbiological analysis.

Isolation of *P. aeruginosa*

Samples were serially diluted up to 10^{-7} with sterile normal saline. Then, 100 μL of each dilution was spread on Cetrimide agar (Oxoid, UK) and incubated overnight at 37 °C. Colonies with *Pseudomonas*-like characteristics were subculture on nutrient agar and subjected to a wide array of biochemical tests (oxidase, catalase, triple sugar iron, indole, citrate utilization, methyl red, Voges-Proskauer, and urease) for presumptive identification.

Molecular identification of *P. aeruginosa*

The identity of presumptively identified *P. aeruginosa* was confirmed by PCR amplification of the *oprL* gene (Table 1). The total reaction mixture was 25 μL, which consisted of 12.5 μL of 2× master mix (Promega, USA), 1 μL of each primer (10 μM forward and reverse each), 8.5 μL of nuclease-free water, and 2 μL of template DNA. The thermocycling conditions were initial denaturation at 95 °C for 5 min, 35 cycles consisting of denaturation at 94 °C for 45 s, annealing at 58 °C for 30 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min. Amplicons were separated in agarose (1.5%) gel electrophoresis.

Antimicrobial susceptibility profiling

The Kirby-Bauer disc diffusion method was followed for the characterization of bacterial resistance to 14 antibiotics including penicillin (10 μg), imipenem (10 μg), meropenem (10 μg), ofloxacin (5 μg), ciprofloxacin (5 μg), co-trimoxazole (25 μg),

Table 1 | List of primers with the amplicon size and the annealing temperature used in the study

Target genes	Primer	Primer sequence	Amplicon size (bp)	Annealing temperature	References
oprL	F (5′ → 3′) R (5′ → 3′)	ATGGAAATGCTGAAATTCGGC CTTCTTCAGCTCGACGCGACG	504	58 °C	Chand *et al.* (2021)
algD	R (5′ → 3′) R (5′ → 3′)	CTACATCGAGACCGTCTGCC GCATCAACGAACCGAGCATC	593	58 °C	Banar *et al.* (2016)
pelf	F (5′ → 3′) R (5′ → 3′)	GAGGTCAGCTACATCCGTCG TCATGCAATCTCCGTGGCTT	789	52 °C	
pslD	F (5′ → 3′) R (5′ → 3′)	TGTACACCGTGCTCAACGAC CTTCCGGCCCGATCTTCATC	369	52 °C	
Int1	F (5′ → 3′) R (5′ → 3′)	CAGTGGACATAAGCCTGTTC CCCGAGGCATAGACTGTA	160	54 °C	Aryanezhad *et al.* (2016)
Int2	F (5′ →3′) R (5′ →3′)	CACGGATATGCGACAAAAAGGT GTAGCAAACGAGTGACGAAATG	789	56 °C	
Int3	F (5′ →3′) R (5′ →3′)	GCCTCCGGCAGCGACTTTCAG ACGGATCTGCCAAACCTGACT	980	55 °C	

aztreonam (30 μg), gentamicin (30 μg), amikacin (30 μg), teicoplanin (30 μg), erythromycin (15 μg), ceftazidime (30 μg), colistin(10 μg), and chloramphenicol (30 μg) (Oxoid, UK) (Bauer *et al.* 1966). Isolates were grown in Mueller Hinton Broth and turbidity was adjusted to 0.5 McFarland standards. A uniform lawn was prepared by spreading culture on Mueller Hinton Agar (Oxoid, UK) using sterile cotton swabs. Antibiotic discs were placed on the surface of the media and incubated at 37 °C for 24 h. The diameter of the zone of inhibition was measured and classified as sensitive, intermediate, or resistant in accordance with the CLSI guidelines (CLSI 2023). Isolates that showed resistance to three or more classes of antibiotics were considered as MDR. Moreover, the number of antibiotics to which isolates were resistant was divided by the number of total antibiotics to calculate the multiple antibiotic resistance (MAR) index (Davis & Brown 2016).

Biofilm formation assay

The detection and quantification of bacterial biofilm-forming ability were done by the crystal violet microtiter plate assay. Isolates were inoculated in Luria Bertani (LB) broth and incubated overnight at 37 °C. The bacterial culture turbidity was adjusted to 1 McFarland standard using the fresh LB medium. Then, 200 μL of culture was inoculated into a 96-well flat-bottom microtiter plate (Corning, USA). Following incubation at 37 °C for 24 h, the planktonic cells were washed out with water. Then, the staining of biofilm was done by adding 200 μL of 0.1% crystal violet to the microtiter plate. After washing the stain with water, the biofilm was dissolved in 200 μL of 96% ethanol. Finally, the absorbance was taken at 492 nm using the microplate reader (Promega, USA). A test medium without cells was used as a negative control. Then, the absorbance of the test samples was compared with that of the control. This was followed by classifying the extent of biofilm formation as either no, weak, moderate, or strong, as previously described (Stepanović *et al.* 2007).

DNA extraction

Chromosomal DNA of isolates was extracted using the boiling lysis method (De Medici *et al.* 2003). This method involves growing bacteria in nutrient broth, centrifuging at 10,000 rpm for 5 min, dissolving the bacterial pellet in nuclease-free water, heating the bacterial suspension at 100 °C for 10 min, and immediately transferring onto ice. The suspension is then centrifuged again at 10,000 rpm for 10 min, and finally, the supernatant (80 μL) containing DNA is collected. The purity and quantity of extracted DNA were measured using NanoDrop and stored at −20 °C.

Detection of biofilm and integron-related genes

The polymerase chain reaction was carried out using the gene-specific primer to detect biofilm (*algD*, *pelf*, and *pslD*) and integron-related genes (*int1*, *int2*, and *int3*). The reaction volume was 25 μL, which consisted of 12.5 μL of 2× master mix (Promega, USA), 1 μL of each primer (10 μM forward and reverse each), 8.5 μL of nuclease-free water, and 2 μL of template DNA. *P. aeruginosa* ATCC 27853 DNA and the distilled water were as used as positive and negative controls, respectively. Primer sequence, amplicon size, and annealing temperature are listed in Table 1. Once thermal cycling (Eppendorf

Mastercycler, Germany) was completed, agarose gel electrophoresis was conducted to separate amplified fragments. EtBr-stained agarose gel was visualized on the AlphaImager Mini Gel Documentation System (ProteinSimple, USA).

Statistical analysis

The statistical analysis used in this study was the chi-square method for the analysis of categorical data among different groups of biofilm producers using GraphPad Prism (Version 8.0). Differences were considered significant if the two-tailed *P*-value was <0.05.

RESULTS

A total of 81 *P. aeruginosa* isolates were recovered from collected wastewater samples based on their selective growth on Cetrimide agar, biochemical profiles, and amplification of the *oprL* gene. Isolates showed varying degrees of sensitivity toward commonly used antibiotics. The overall scenario of antimicrobial resistance is represented graphically in Figure 1. Based on our analysis, 76 out of 81 (93.82%) isolates were found to be MDR. All the isolates (100%) showed resistance to penicillin G and erythromycin followed by co-trimoxazole (94%), teicoplanin (93%), and chloramphenicol (89%). On the other hand, the percentages of isolates that showed sensitivity toward meropenem, gentamicin, and amikacin were 85, 83, and 81%, respectively. The percentage of sensitivity toward ciprofloxacin and ofloxacin was 75%. The degree of sensitivity of isolates toward imipenem, aztreonam, and ceftriaxone varied. Moreover, the calculation of the MAR index revealed that 56.79% of isolates had a MAR score of 0.4. Only four isolates (4.94%) showed resistance to all the used antibiotics.

The crystal violet microtiter plate assay was performed for the qualitative and quantitative measurement of bacterial biofilm formation abilities. The optical density of the control (Control OD = 0.08) and test isolates were compared to determine the extent of biofilm formation (weak, moderate, and strong). We found that 76 of the 81 isolates (93.82%) were biofilm formers. Weak, moderate, and strong biofilm formers corresponded to 48.15, 33.34, and 12.34% of the isolates, respectively (weak = 0.071–0.140, moderate = 0.141–0.280, and strong = >0.280). The remaining isolates (6.18%) showed no biofilm formation activities during 24 h of incubation.

All isolates were subjected to the PCR for the molecular detection of three biofilm-associated genes *algD*, *pelF,* and *pslD* (Table 2). The positive band for *algD* and *pslD* genes was observed in 95% of isolates, whereas the percentage of isolates that showed positive amplification for *pelF* was 90%. All three tested genes were present in 67 (82.7%) isolates, making $algD^{+}pelF^{+}pslD^{+}$ the predominant genotypic pattern (Figure 2). The number of biofilm-forming isolates that lacked any of the genes was 13.

Among 76 MDR isolates, 71 were found to be biofilm former (weak = 37, moderate = 24, and strong = 10). The chi-square test revealed no significant association between multidrug resistance and biofilm formation ability. In addition to biofilm-related genes, integron-associated genes, such as *int1*, *int2*, and *int3*, were also amplified. Sixteen out of 81 (19.75%) isolates

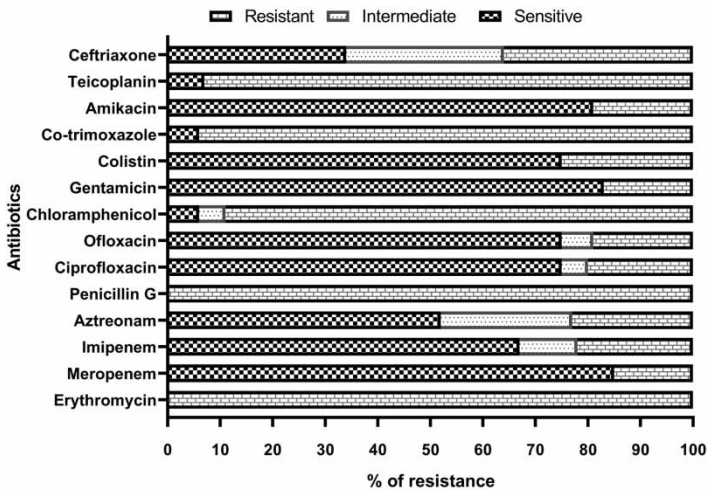


Figure 1 | Graphical representation of the antibiotic susceptibility patterns of *P. aeruginosa*.

Table 2 | Relationship between genotypic and phenotypic patterns of biofilm characteristics among *P. aeruginosa* isolates

	Genotypic pattern of biofilm, *N* (%)	
Phenotypic pattern of biofilm, *N* (%)	**All gene positive (algD$^+$pelF$^+$pslD$^+$)**	**Missing any gene (algD$^-$/ pelf$^-$ /pslD$^-$)**
Strong, 10 (12.34)	9 (11.11)	1 (1.23)
Moderate, 27 (33.34)	22 (27.16)	5 (6.17)
Weak, 39 (48.15)	32 (39.5)	7 (8.65)
No biofilm, 5 (6.17)	4 (4.94)	1 (1.23)
Total, 81 (100)	67 (82.72)	14 (17.28)

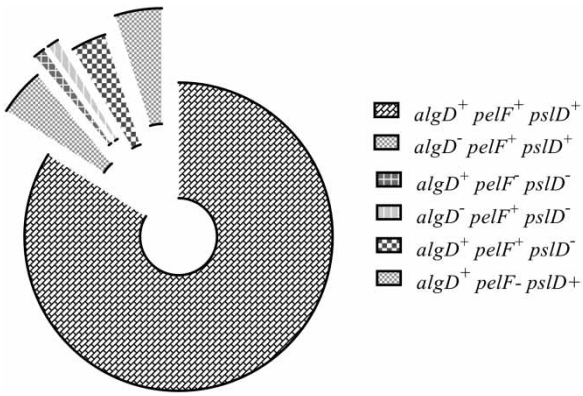


Figure 2 | Distribution pattern of biofilm-related genes among isolates.

were found to carry the *int1* gene in their chromosome, whereas no isolates yielded positive amplification for the *int2* and *int3* genes.

DISCUSSION

Research on antimicrobial resistance and biofilm formation in environmental bacteria is less evident in previous literature compared to clinical isolates, even though infection with environmental isolates has been described (Rabbani *et al.* 2017). Current research suggests that hospital wastewater is one of the most important reservoirs in the environmental dissemination of *P. aeruginosa*. The purpose of the study was to evaluate the antimicrobial resistance patterns and the phenotypic as well as genotypic characteristics of biofilm formation in *P. aeruginosa* strains isolated from hospital wastewater in Dhaka, Bangladesh. In this study, MDR phenotypes were observed in 93.82% of isolates, which presents an alarming situation and reflects the loss of effectiveness of a large number of antibiotics used in hospital settings. This finding is quite consistent with the increasing worldwide occurrence of MDR *P. aeruginosa* in hospital wastewater and related environments (Miranda *et al.* 2015; Divyashree *et al.* 2022; Saha *et al.* 2022). Such high occurrence of MDR might be due to the overuse of antibiotics in hospital settings that are discharged in the hospital effluent, triggering the emergence of resistance. Isolates showed diverse extents of susceptibility toward different classes of antibiotics. The effectiveness of meropenem, gentamicin, and amikacin is comparable (81–85%). About 85% of isolates showed sensitivity toward meropenem. This finding is in agreement with other reports that determined 80–95% sensitivity toward meropenem in *P. aeruginosa* in South Africa and the Czech Republic (Miranda *et al.* 2015; Mapipa *et al.* 2021; Roulová *et al.* 2022). The sensitivity of wastewater isolates of *P. aeruginosa* to amikacin and gentamicin is also evident in the existing literature (Moges *et al.* 2014; Mapipa *et al.* 2021; Roulová *et al.* 2022).

Ciprofloxacin is one of the most used fluoroquinolones that play an irreplaceable therapeutic role against a wide variety of *P. aeruginosa* infections (Rehman *et al.* 2019). Therefore, resistance to this antibiotic can cause serious complications in the therapeutic strategy. In this study, 20% of isolates exhibited resistance to both fluoroquinolones (ciprofloxacin and ofloxacin). *P. aeruginosa* is known to employ multifactorial mechanisms including mutation at quinolone resistance-determining regions, HGT-mediated acquisition of resistance gene, efflux-mediated expulsion of antibiotics, and others to become

ciprofloxacin-resistant (Pang *et al.* 2019). We observed that the proportions of isolates that exhibited reduced susceptibility and resistance to aztreonam were 25 and 23%, respectively, indicating the increasing prevalence of aztreonam resistance among isolates. Santoro *et al.* (2012) found decreased susceptibility to aztreonam in 62.9% of *P. aeruginosa* from hospital sewage, whereas the percentage of resistance was higher among clinical isolates. Such variation in occurrence might depend on exposure characteristics and adopting mechanisms of action in clinical and wastewater ecosystems. All the isolates exhibited resistance to penicillin G and erythromycin. The high occurrence of isolates with resistance to co-trimoxazole (94%), teicoplanin (93%), and chloramphenicol (89%) indicates that these antibiotics are losing their effectiveness in controlling *P. aeruginosa*.

Biofilm formation ability was detected in 93.82% of isolates, of which 48.15% showed weak biofilm formation activities. The genotypic characterization of biofilm formation showed a high prevalence of three biofilm-related genes, such as *algD* (95%), *pslD* (95%), and *pelF* (90%). Such high prevalence is documented in clinical isolates of *P. aeruginosa* (Yang *et al.* 2021; Rajabi *et al.* 2022). The frequency of $algD^{+}$pelF^{+}pslD^{+} among biofilm former isolates was 82.7%. A similar pattern was also observed among 87.5% of *P. aeruginosa* isolates as reported by Kamali *et al.* (2020). In contrast, 13 isolates that did not yield a positive band for any of the three genes also produced different degrees of biofilm formation in the microtiter plate assay. We did not find any association ($\chi^2 = 0.43$, $P = 0.93$) between the presence or absence of any tested genes with biofilm formation. The involvement of other biofilm-associated genes might be responsible for this inconsistency (Friedman & Kolter 2004; Moradali *et al.* 2017; Abdelraheem *et al.* 2020). In addition, despite the presence of genes, mutation in numerous regulatory components can affect biofilm formation (Hou *et al.* 2012).

Integrons are often associated with AMR genes and therefore have a role in the environmental dissemination of resistance via wastewater (Stalder *et al.* 2012). The integrase 1 gene was found to be present in 19.75% (16 out of 81) of isolates, but no isolates possessed *int2* or *int3*. All integron 1-carrying isolates were MDR except one in the current study. A high rate of MDR *P. aeruginosa* was reported among integron-positive isolates from wastewater of a burn center in Iran (Ebrahimpour *et al.* 2018). Similar findings were also reported in *Escherichia coli* from a wastewater treatment plant in Dhaka, Bangladesh (Hossain *et al.* 2022).

The ability of biofilm to spread resistance genes and its inherent phenotypic tolerance to antibiotics drive researchers to consider it synonymously with antibiotic resistance (Bowler *et al.* 2020). However, the literature provides contrasting evidence concerning the connection between biofilm and antimicrobial resistance in *P. aeruginosa*. According to this study, multidrug resistance had no significant associations with biofilm-producing abilities ($\chi^2 = 0.35$, $P = 0.55$). Our result is in concordance with other findings (Cepas *et al.* 2019; Davarzani *et al.* 2021; Gajdács *et al.* 2021; Behzadi *et al.* 2022). In contrast, several studies have highlighted the association between multidrug resistance and biofilm production (Gurung *et al.* 2013; Magalhães *et al.* 2016; Abdulhaq *et al.* 2020; Kamali *et al.* 2020). This disagreement might be attributed to the number and types of samples, geographical variation in pathogen prevalence, factors affecting biofilm formation, type and frequency of antibiotics used, the involvement of other resistance mechanisms including efflux pumps, alteration in membrane permeability, and beta-lactamase.

CONCLUSION

This study reports the present trends of antibiotic resistance among wastewater isolates of *P. aeruginosa* along with their biofilm formation ability in Dhaka city, Bangladesh. Most of the isolates were MDR and biofilm-forming. The lack of data on the molecular detection of AMR genes is one of the major limitations of the study. More research on the extent of the transmission of MDR *P. aeruginosa* in hospital-surrounding environments and biofilm-mediated AMR development is essential to determine the public health importance of this pathogen. In addition, whole genome sequencing might provide valuable data on the genetic basis of resistance, emerging resistant clones that can be combinedly used for the environmental surveillance of *P. aeruginosa*.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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