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Original article

Correlation Between Virulence Determinants and Multidrug Resistance in *Pseudomonas aeruginosa* Isolated from Healthcare-Associated Infections in Libya

Muhammad Elrahait[1], Amal Henaish[2], Mohanned Alwashaish[*1]

[1]*Department of Biomedical Science, Faculty of Pharmacy, University of Misurata, Misurata, Libya*
[2]*Department of Microbiology, Faculty of Science, University of Misurata, Misurata, Libya*
Corresponding Email. *mohanad.aloshesh@phar.misuratau.edu.ly*

Abstract
Pseudomonas aeruginosa is a major cause of healthcare-associated infections, combining multidrug resistance (MDR) with virulence traits that complicate therapy, prolong hospitalization, and increase mortality. This study aimed to assess the prevalence of key virulence determinants and antimicrobial resistance in *P. aeruginosa* isolates from Libya, and to examine their associations. A total of 179 isolates were collected from urine, wound, respiratory, and blood samples from healthcare settings. Virulence factors (biofilm, hemolysin, protease, pyocyanin) were evaluated phenotypically. Antimicrobial susceptibility was tested by the Kirby–Bauer disk diffusion method following CLSI 2023 guidelines. Statistical tests determined associations between resistance and virulence. Biofilm was the most common virulence trait (72.6%; strong: 38.5%, moderate: 34.1%), followed by hemolysin (61.5%), protease (57.5%), and pyocyanin (48.0%). Resistance was highest to ceftazidime (69.3%), ciprofloxacin (62.6%), and gentamicin (58.1%), while colistin (92.7% susceptible) and meropenem (76.5% susceptible) remained most effective. Biofilm-producing isolates showed significantly higher resistance than non-producers (x^2=9.81, p=0.002, effect size=0.24). This study is the first in Libya to simultaneously link *P. aeruginosa* virulence with multidrug resistance (MDR). The coexistence of biofilm formation and resistance underscores its clinical importance in treatment failure and persistent infections. These findings highlight the urgent need to integrate virulence monitoring into antimicrobial resistance surveillance and to strengthen stewardship and infection-control programs in regional healthcare systems. Furthermore, incorporating virulence profiling into AMR surveillance may enhance infection control strategies and guide empirical therapy in Libyan hospitals.
Keywords. *Pseudomonas aeruginosa*, Hospital-Acquired Infections, Virulence Factors, Multidrug Resistance, Biofilm.

Introduction

Healthcare-associated infections (HAIs) are a major global health concern, causing prolonged hospitalization, morbidity, and mortality [1]. Among Gram-negative pathogens, *Pseudomonas aeruginosa* is particularly problematic due to its intrinsic and acquired resistance mechanisms, combined with multiple virulence factors that facilitate persistence in clinical environments [2–4]. It commonly causes urinary tract, wound, respiratory (particularly ventilator-associated pneumonia), and bloodstream infections [5]. Key virulence determinants include biofilm formation, hemolysins, proteases, and pyocyanin, which collectively enhance bacterial survival, colonization, and tissue damage [6–8]. In parallel, *P. aeruginosa* frequently exhibits multidrug resistance (MDR) to β-lactams, aminoglycosides, and fluoroquinolones, limiting effective therapeutic options to carbapenems and colistin [9]. The coexistence of virulence and resistance complicates clinical management and poses challenges for infection control, especially in resource-limited settings [10]. Although extensive data exist from Europe and North America, information from North Africa is limited. Regional studies often examined either resistance or virulence separately, without evaluating their combined impact [11–13].

To our knowledge, this is the first comprehensive study from Libya to simultaneously assess both antimicrobial resistance and virulence determinants of *P. aeruginosa* clinical isolates [14]. By investigating the association between virulence and resistance, this work provides novel evidence from a region with strong epidemiological connections to Europe, thereby contributing to both local and international surveillance strategies [15,16].

Methods

Study design

This cross-sectional study was conducted at the Microbiology laboratory of the Faculty of Science. A total of 179 non-duplicate *Pseudomonas aeruginosa* isolates were collected from various clinical specimens between June 2024 – March 2025.

Clinical specimens and bacterial identification

Clinical specimens, including urine, wound swabs, respiratory samples, and blood cultures, were obtained from hospitalized patients with suspected healthcare-associated infections. Initial identification of isolates was performed using standard microbiological methods, such as colony morphology assessment, Gram

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staining, and oxidase testing. Definitive identification was conducted with the API 20NE system (bioMérieux, France) according to the manufacturer's protocols. Pseudomonas aeruginosa ATCC 27853 was employed as a quality control strain throughout the study [17].

Assessment of Virulence Factors

The production of key virulence factors was assessed for all isolates. Hemolysin activity was evaluated by streaking isolates onto 5% sheep blood agar plates and incubating them aerobically at 37°C for 24 hours; a positive result was indicated by the presence of a clear or greenish zone of hemolysis around the colonies [18]. Protease production was determined using skim milk agar plates, where the formation of a clear hydrolytic zone surrounding colonies after 24 hours of incubation at 37°C confirmed activity [19]. To detect pyocyanin production, isolates were cultured on King's A medium at 37°C for 24 hours; the pigment was then extracted with chloroform and quantified spectrophotometrically at 690 nm [20].

Biofilm formation was evaluated using two complementary methods. The tube adherence method involved growing isolates in brain–heart infusion broth at 37°C for 24 hours. The resulting biofilms were washed, stained with crystal violet, and considered positive if a visible film adhered to the tube wall. The Congo red agar (CRA) assay was used as a confirmatory test, with black colonies indicating strong biofilm production [21, 22]. All virulence factor assays were performed in triplicate to ensure reproducibility.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar, in accordance with the Clinical and Laboratory Standards Institute (CLSI) 2023 guidelines [23]. The following antibiotic agents from major classes were evaluated: β-lactams (ceftazidime, piperacillin–tazobactam), carbapenems (imipenem, meropenem), aminoglycosides (gentamicin, amikacin), fluoroquinolones (ciprofloxacin), and polymyxins (colistin). However, for colistin, susceptibility was determined using the broth microdilution (BMD) method, as it is the CLSI-recommended reference standard for polymyxins.

Results were interpreted based on CLSI breakpoints. Pseudomonas aeruginosa ATCC 27853 was used as the quality control strain for all antimicrobial tests. Isolates were classified as multidrug-resistant (MDR) if they demonstrated non-susceptibility to at least one agent in three or more antimicrobial categories [24].

Statistical analysis

Data were analyzed using SPSS version 25 (IBM Corp., Armonk, NY, USA). Associations between virulence factors and resistance profiles were assessed using the Chi-square test or Fisher's exact test where appropriate. Differences in mean resistance across groups were analyzed using one-way ANOVA or Kruskal–Wallis tests for non-parametric data. Effect sizes were estimated using Cramer's V and eta squared. A p-value <0.05 was considered statistically significant [25].

Results

Distribution of isolates by specimen type

Among the 179 isolates of *Pseudomonas aeruginosa*, the highest proportion was obtained from urine samples (n=65, 36.3%), followed by wound swabs (n=54, 30.2%), respiratory secretions (n=38, 21.2%), and blood cultures (n=22, 12.3%) as revealed in (Table 1). Chi-square analysis indicated a significant variation in distribution across specimen types (x^2=24.5, df =3, p<0.001, Cramer's V=0.37, large effect size). Post-hoc pairwise comparisons (adjusted residuals >±1.96) revealed that urine isolates were significantly more common, while blood isolates were significantly fewer than expected.

Table 1. Distribution of P. aeruginosa isolates according to specimen type

Specimen type	No. of isolates (n=179)	Percentage (%)
Urine	65	36.3
Wound swabs	54	30.2
Respiratory samples	38	21.2
Blood	22	12.3

Virulence factors of P. aeruginosa

Out of the 179 *P. aeruginosa* isolates, biofilm formation was the most frequently observed virulence trait (72.6%). Among these, 38.5% were strong producers, 34.1% moderate, while 27.4% exhibited weak or no biofilm activity. Hemolysin production was detected in 110 isolates (61.5%), protease activity in 103 isolates (57.5%), and pyocyanin production in 86 isolates (48.0%), as shown in (Table 2). Chi-square analysis demonstrated significant variation in the prevalence of virulence factors (x^2=22.3, df=3, p<0.001, Cramer's V=0.35, medium effect size). The Kruskal–Wallis test revealed significant differences in mean resistance scores across biofilm categories (H = 10.24, df = 2, p = 0.006, η^2 = 0.07). Pairwise comparisons revealed

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significantly higher resistance among strong biofilm producers compared with weak/non-producers (p = 0.004).

Table 2. Distribution of virulence factors among P. aeruginosa isolates

Virulence factor	Positive isolates (n)	Percentage (%)
Biofilm formation		
Strong	69	38.5
Moderate	61	34.1
Weak/none	49	27.4
Total biofilm-positive	130	72.6
Hemolysin	110	61.5
Protease	103	57.5
Pyocyanin	86	48.0

Antimicrobial resistance patterns

Antimicrobial susceptibility testing of the 179 *P. aeruginosa* isolates revealed high levels of resistance across multiple antibiotic classes. The highest resistance rates were observed against ceftazidime (124/179, 69.3%), ciprofloxacin (112/179, 62.6%), and gentamicin (104/179, 58.1%). Moderate resistance levels were found against amikacin (46.4%) and piperacillin–tazobactam (54.2%). Resistance to carbapenems was relatively lower, with 34.1% of isolates resistant to imipenem and 23.5% resistant to meropenem. Colistin remained the most effective agent, with only 7.3% of isolates exhibiting resistance, as presented in (Table 3 and Figure 1). Chi-square test confirmed significant variation in resistance across antibiotic categories (x^2=131.7, df=7, p<0.001, Cramer's V=0.61, large effect size). One-way ANOVA indicated significant differences in mean resistance rates between antibiotic groups (F=15.42, df=3, p<0.001, η^2=0.21). Post-hoc analysis (Tukey HSD) showed resistance to ceftazidime and ciprofloxacin was significantly higher than to meropenem and colistin (p<0.01).

Table 3. Antimicrobial resistance among P. aeruginosa isolates

Antibiotic	Resistant n (%)	Intermediate n (%)	Susceptible n (%)
Ceftazidime	124 (69.3)	18 (10.1)	37 (20.6)
Ciprofloxacin	112 (62.6)	21 (11.7)	46 (25.7)
Gentamicin	104 (58.1)	19 (10.6)	56 (31.3)
Amikacin	83 (46.4)	25 (14.0)	71 (39.7)
Piperacillin-tazobactam	97 (54.2)	22 (12.3)	60 (33.5)
Imipenem	61 (34.1)	17 (9.5)	101 (56.4)
Meropenem	42 (23.5)	17 (9.5)	120 (67.0)
Colistin	13 (7.3)	0 (0.0)	166 (92.7)

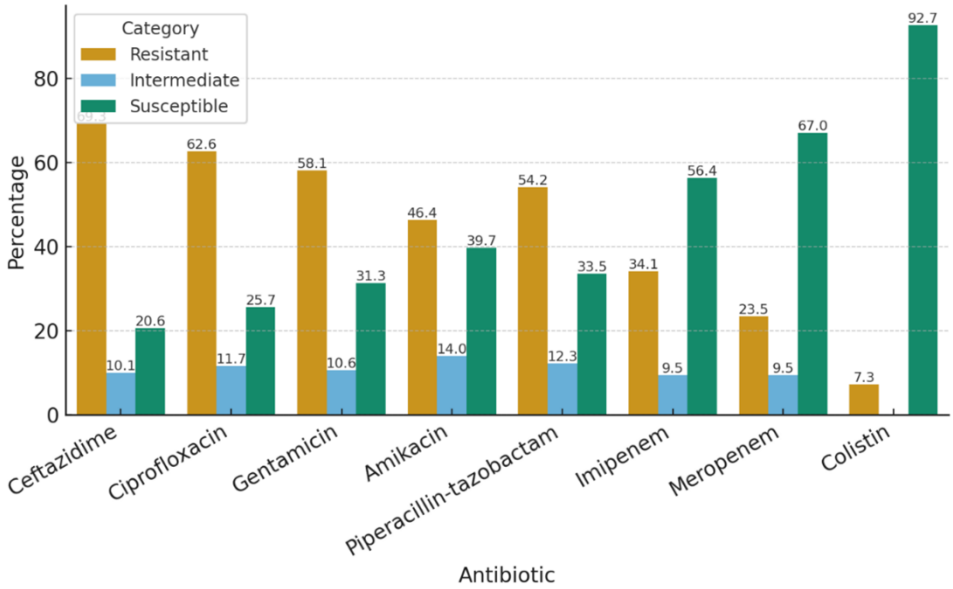

Figure 1. Antimicrobial resistance profile of P. aeruginosa isolates

Bar chart showing the percentage of resistant, intermediate, and susceptible isolates across eight tested antibiotics.

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Association between biofilm formation and multidrug resistance (MDR)

Out of the 179 *P. aeruginosa* isolates, 102 (57.0%) were classified as multidrug resistant (MDR), 21 (11.7%) as extensively drug-resistant (XDR), and 56 (31.3%) as non-MDR. Biofilm-producing isolates (n=130, 72.6%) demonstrated markedly higher rates of MDR (65.4%) compared to non-biofilm producers (34.7%). When categorized by biofilm strength, MDR prevalence was highest among strong biofilm producers (78.3%), followed by moderate (50.8%) and weak/non-biofilm isolates (34.7%), as illustrated in (Table 4 and Figure 2). Chi-square analysis indicated a significant association between biofilm formation and MDR status (x^2=9.81, df=2, p=0.002, Cramer's V=0.24, medium effect size). One-way ANOVA showed significant differences in mean resistance scores across biofilm strength groups (F=4.92, df=2, p=0.008, η^2=0.07). Tukey post-hoc analysis revealed that strong biofilm producers were significantly more resistant than weak/non-producers (p=0.004).

Table 4. Association between biofilm formation and multidrug resistance in P. aeruginosa

Biofilm category	MDR n (%)	Non-MDR n (%)	Total n
Strong biofilm	54 (78.3)	15 (21.7)	69
Moderate biofilm	31 (50.8)	30 (49.2)	61
Weak/none	17 (34.7)	32 (65.3)	49
Total	102 (57.0)	77 (43.0)	179

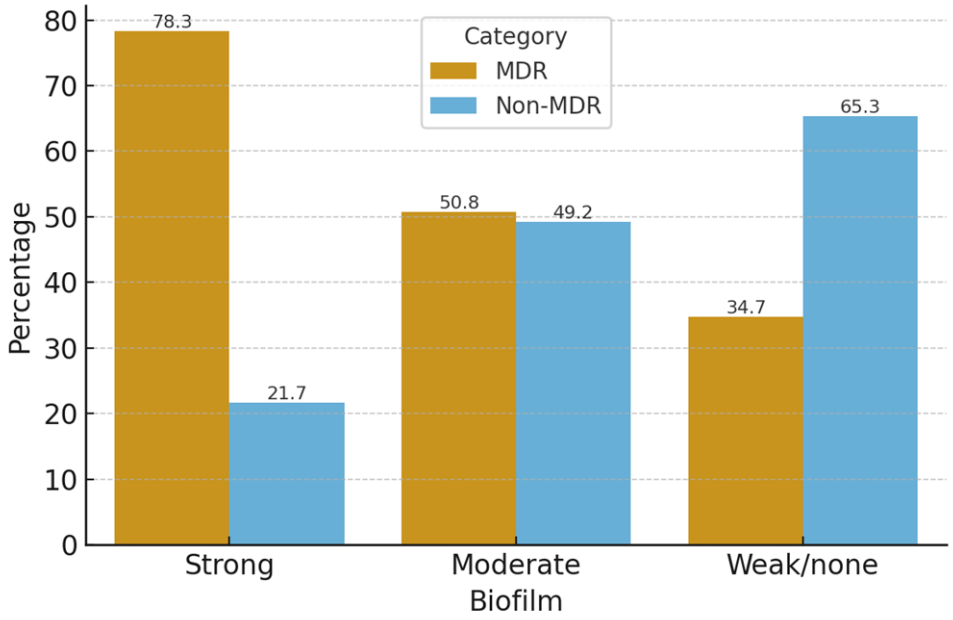

Figure 2. Relationship between biofilm strength and MDR status in P. aeruginosa

Clustered bar chart showing the distribution of MDR and non-MDR isolates across biofilm categories (strong, moderate, weak/none).

Association between virulence factors and antimicrobial resistance

When analyzing the relationship between virulence traits and resistance across all tested antibiotics, significant patterns emerged. Biofilm-forming isolates consistently demonstrated higher resistance rates compared to non-biofilm producers, particularly against ceftazidime (75.4% vs. 58.2%), ciprofloxacin (68.5% vs. 51.0%), and gentamicin (63.8% vs. 46.9%). Hemolysin-positive isolates showed notably higher resistance to ceftazidime (74.5% vs. 61.4%) and piperacillin–tazobactam (63.6% vs. 42.0%). Protease-positive isolates exhibited increased resistance to ciprofloxacin (68.0% vs. 55.3%) and aminoglycosides. In contrast, pyocyanin production did not show significant associations with resistance to any antibiotic, with resistance levels similar to pyocyanin-negative isolates across the board, as shown in (Table 5 and Figure 3). Chi-square tests demonstrated significant associations between biofilm formation and resistance to ceftazidime (x^2=9.81, p=0.002, Cramer's V=0.24), ciprofloxacin (x^2=7.12, p=0.008, Cramer's V=0.20), and gentamicin (x^2=6.54, p=0.011, Cramer's V=0.19). Hemolysin positivity was associated with resistance to ceftazidime (x^2=4.15, p=0.042, Phi=0.15) and piperacillin–tazobactam (x^2=6.38, p=0.012, Phi=0.19). Protease activity correlated with ciprofloxacin resistance (Kruskal–Wallis H=4.66, p=0.031, η^2=0.08). Pyocyanin production showed no significant associations with resistance to any antibiotic (all p>0.05). Heatmap illustrating resistance rates among P. aeruginosa isolates according to virulence traits (biofilm, hemolysin, protease, pyocyanin). Darker shades represent higher resistance percentages.

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Table 5. Association between virulence factors and antimicrobial resistance in P. aeruginosa

Antibiotic	Biofilm (+) Resistant %	Hemolysin (+) Resistant %	Protease (+) Resistant %	Pyocyanin (+) Resistant %
Ceftazidime	75.4	74.5	70.0	73.3
Ciprofloxacin	68.5	65.4	68.0	63.9
Gentamicin	63.8	61.8	60.2	59.1
Amikacin	55.4	53.6	56.3	50.5
Piperacillin–tazobactam	60.8	63.6	58.3	55.5
Imipenem	38.5	36.4	39.8	35.5
Meropenem	27.7	28.2	30.1	24.4
Colistin	9.2	8.2	10.7	7.8

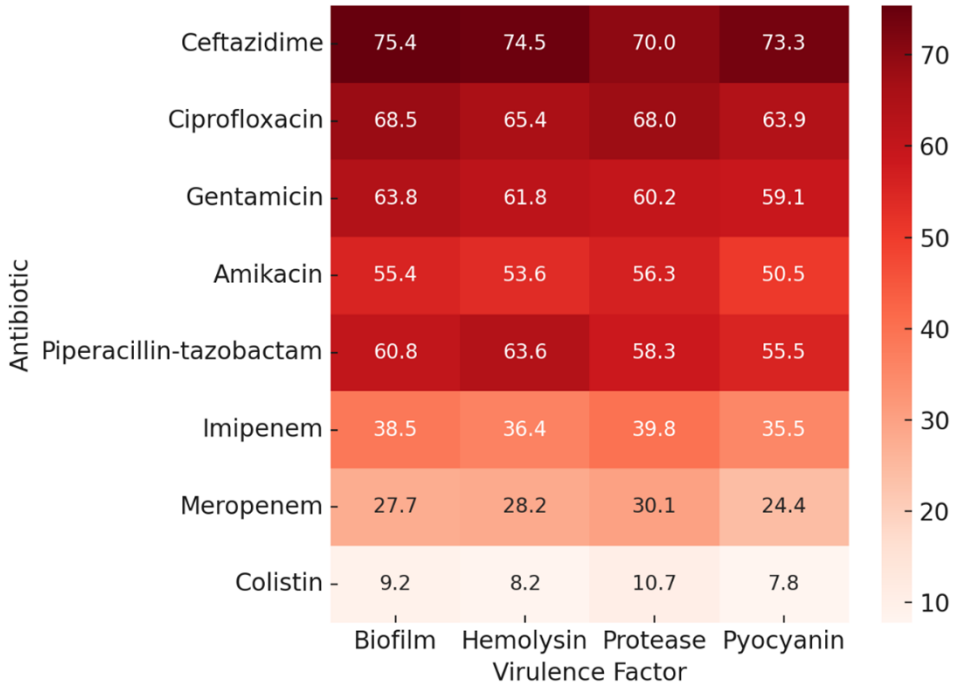

Figure 3. Heatmap of associations between virulence factors and antibiotic resistance

Discussion

This study provides one of the first comprehensive evaluations of *Pseudomonas aeruginosa* in Libya, examining both antimicrobial resistance patterns and key virulence determinants. The coexistence of MDR with high prevalence of virulence factors underscores the pathogen's dual threat to patient outcomes and infection control strategies. Urinary isolates were the most frequent, consistent with the fact that urinary tract infections (UTIs) represent one of the leading healthcare-associated infections (HAIs) worldwide [1]. Similar proportions have been reported in European surveillance where UTIs accounted for a large share of *P. aeruginosa* HAIs [26,27]. Wound isolates reflected the pathogen's well-documented role in burn and surgical site infections [28]. Respiratory and bloodstream isolates were less common but remain clinically critical, as bloodstream infections are associated with high mortality in hospital cohorts [29]. The predominance of urinary isolates can be explained by frequent use of catheters, which provide a surface for biofilm adherence [14–16]. Wound and respiratory isolates mirror the exposure of injured tissue and ventilated lungs to hospital reservoirs of *P. aeruginosa*. Bloodstream infections, though less frequent, indicate systemic spread from primary foci and therefore carry the highest fatality risk.

High biofilm production of isolates, in line with earlier reports where prevalence exceeded 70% [14–16]. Strong biofilm producers showed a significant association with MDR, consistent with studies that demonstrated biofilms act as protective niches and promote horizontal gene transfer [30–32]. Within biofilms, antibiotics cannot penetrate effectively, and metabolically inactive, persistent cells remain unaffected by drug action. These mechanisms explain the persistence of catheter-associated UTIs and ventilator-associated pneumonia despite treatment [33]. Hemolysin and protease were also frequent and correlated with resistance to β-lactams and fluoroquinolones. European studies have suggested that expression of these virulence factors often overlaps with resistance mechanisms [12,31]. Hemolysins damage host membranes and liberate iron, while proteases degrade host immune proteins. When combined with resistance, they amplify severity: the pathogen not only survives therapy but also inflicts greater tissue damage. Pyocyanin was observed in half of the isolates, without significant correlation to resistance. This matches findings that pyocyanin contributes to oxidative stress and host tissue damage, but is regulated

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independently from resistance pathways [13,31].

Unlike biofilm or proteases, pyocyanin production is primarily under quorum-sensing control. Its biological role is to modulate host immunity and inter-bacterial competition, not to mediate resistance, explaining the lack of statistical association. Highest resistance to ceftazidime and ciprofloxacin, followed by gentamicin. Similarly high rates to these agents have been documented in North African and European hospitals [26,27]. Moderate resistance to carbapenem, echoing findings from Tunisia and the Mediterranean basin [32,33]. Colistin remained highly effective, with only low-resistant isolates, consistent with global observations that colistin retains activity against *P. aeruginosa* [34]. Ceftazidime and ciprofloxacin are frequently prescribed empirically, creating strong selective pressure. Carbapenems are more restricted, but the emergence of OXA-48 and other carbapenemases has driven resistance in the region [32,33]. Colistin's effectiveness reflects its limited use as a last-line drug, but the global dissemination of *mcr* genes threatens its reliability [10,22].

This highlights the urgent need for novel therapeutic strategies, including β-lactam/β-lactamase inhibitor combinations, anti-biofilm agents, and bacteriophage therapy, which have shown promising results in overcoming MDR *P. aeruginosa*.

The strong correlation between biofilm formation and MDR underscores the role of biofilms in treatment failure [30–32]. ICU studies have similarly shown worse outcomes when infections involve biofilm-forming *P. aeruginosa* [33]. Hemolysin and protease correlations with resistance reinforce the idea that virulence and resistance can coexist synergistically [12,31]. Pyocyanin's lack of association confirms that virulence factors differ in their contribution to resistance [13,31]. In many pathogens, the acquisition of resistance comes with a fitness cost. *P. aeruginosa* is exceptional: resistant strains often maintain or even enhance virulence. This dual adaptation explains its persistence in hospital reservoirs and its critical status in global AMR lists [34]. The coexistence of MDR and virulence factors explains why *P. aeruginosa* is designated a critical-priority pathogen by the WHO [34]. Clinically, this combination contributes to poor outcomes, prolonged hospitalization, and increased healthcare costs. Cassini *et al.* demonstrated that MDR *P. aeruginosa* accounted for substantial disability-adjusted life years (DALYs) across Europe [35]. From a public health perspective, Libya's geographical and clinical links to Europe highlight the importance of harmonized regional surveillance [36]. Updated EUCAST guidance further stresses the need to adapt therapeutic breakpoints to regional resistance data [37]. Current AMR surveillance usually tracks resistance alone; however, incorporating virulence determinants such as biofilm, hemolysin, and protease would allow clinicians to identify infections at the highest risk of treatment failure. This approach could support early isolation, targeted infection-prevention bundles, particularly in ICUs and surgical wards, and more effective optimization of antimicrobial therapy in high-burden settings.

Future studies should expand surveillance beyond a single center and include molecular methods to identify high-risk clones such as ST111 and ST235 [28,38]. Translational research targeting biofilm disruption, quorum-sensing inhibition, and phage therapy is promising for overcoming MDR [39,40]. Whole-genome sequencing links resistance phenotypes to specific epidemic clones, enabling outbreak detection. Novel approaches like phage therapy and anti-biofilm strategies bypass conventional resistance and provide alternative therapeutic pathways.

This study has some limitations. First, it was a single-city study, which restricts generalizability. Second, only phenotypic assays were performed; molecular characterization of resistance and virulence genes was not included. Third, patient outcome data (mortality, hospital stay) were not captured. Finally, the cross-sectional design prevented assessment of temporal trends. Future research should address these gaps through multi-city surveillance, genomic sequencing, and linking microbiological data with clinical outcomes [41–43].

Conclusion

This is the first Libyan study to simultaneously link antimicrobial resistance with virulence determinants in *P. aeruginosa*, highlighting its dual challenge of resistance and virulence. The high prevalence of biofilm, hemolysin, and protease alongside MDR emphasizes its clinical significance. Clinically, these findings underscore the need for strict infection-prevention measures, rational antimicrobial use, and stewardship programs to reduce unnecessary prescriptions of ceftazidime and fluoroquinolones. From a public health perspective, integrating virulence data into AMR surveillance could strengthen both national and regional strategies [44,45].

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Conflicts of Interest

The author declares no competing interests.

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