JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Phenotypic and molecular analysis of *Pseudomonas aeruginosa* virulence and association with antibiotic resistance in Egypt

Salah Al Ali[1], Aya A Ghamry[2], Amal M Soliman[3], Abdullah M Abdo[4], Nagwan G El-Menofy[5], Manal M Al-Gerby[1], Haytham K Mahrous[1]

[1] *Clinical Pathology Department, Faculty of Medicine, Zagazig University, Sharqia, Egypt*
[2] *Medical Microbiology and Immunology Department, Faculty of Medicine (for Girls), Al-Azhar University, Cairo, Egypt*
[3] *Medical Microbiology and Immunology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt*
[4] *Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt*
[5] *Microbiology and Immunology Department, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt*

Abstract

Introduction: *Pseudomonas aeruginosa* is a common nosocomial pathogen with multidrug resistance (MDR) and virulence factors (VFs). This study assessed the VFs and their associations with MDR and non-MDR isolates.

Methodology: One hundred clinical isolates were analyzed for 12 VFs, encoding genes, and phenotypic traits. Antibiotic resistance patterns and correlations between VFs and MDR were investigated.

Results: Aztreonam showed the highest resistance rate among MDR (94.7%) and ceftazidime showed the highest resistance rate among non-MDR isolates (44.2%). Carbapenems demonstrated the greatest susceptibility. VF positivity rates included 91% for *algD*, 90% for *lasB*, 86% for *toxA*, 82% for *exoS*, 19% for *exoU*, 78% for *aprA*, 75% for *plcH*, 94% for pigment production, 93% for biofilm formation, 72% for hemolysin, 65% for lipase, and 36% for DNase. Strong biofilm formation correlated with *algD* and *lasB* (93%). Pigment production was linked with *lasB* and *toxA* (94%). Strong biofilm formation was significantly higher in MDR isolates and resistant strains, than non-MDR isolates. No significant differences in VFs were observed between susceptible and resistant strains for *lasB*, *algD, toxA*, *plcH*, *exoU*, or general biofilm production; except for strong biofilm formation. Certain VFs correlated with susceptible isolates: *exoS* with tobramycin, *aprA* with aztreonam and piperacillin-tazobactam, pigment production with imipenem, DNase with aztreonam and norfloxacin, and lipase with tobramycin and ceftazidime.

Conclusions: *P. aeruginosa* isolates displayed diverse VFs, biofilm-forming abilities, and MDR profiles; with strong biofilm formation closely linked to MDR. Targeting biofilm-related genes (*algD*, *lasB*) could offer effective therapeutic interventions, helping mitigate MDR infections and improve clinical outcomes.

Key words: *P. aeruginosa*; virulence; genotypic; resistance; nosocomial infections.

J Infect Dev Ctries 2025; 19(5):712-722. doi:10.3855/jidc.20957

(Received 13 October 2024 – Accepted 14 February 2025)

Introduction

Pseudomonas aeruginosa (*P. aeruginosa)* is a Gram-negative opportunistic pathogen and a major cause of healthcare-associated infections. It causes a range of severe infections, including urinary tract, respiratory tract, skin, soft tissue, bone, joint, bloodstream, and systemic infections; particularly in immunocompromised patients such as those with cancer, burns, acquired immunodeficiency syndrome (AIDS), or cystic fibrosis (CF) [1–3]. Serious bloodstream (bacteremia) and lung (pneumonia) infections are associated with high morbidity and mortality, particularly in hospitalized patients. Chronic lung infections in CF patients are driven by the ability of *P. aeruginosa* to form thick, mucoid biofilms, which shield the bacteria from immune responses and antimicrobial treatments, leading to lifelong complications [4–6].

P. aeruginosa thrive in diverse environments, including hospital settings like sinks and ventilators, as well as natural habitats such as soil and water [8,9]. This adaptability is facilitated by its large genome, encoding metabolic pathways and regulatory networks that enable survival across niches [10,11].

Antibiotic resistance in *P. aeruginosa* presents significant challenges, driven by intrinsic, acquired, and adaptive mechanisms. Intrinsic resistance arises from its low outer membrane permeability and efflux pumps that expel antibiotics [12,13]. Acquired resistance is mediated by horizontal gene transfer, where resistance

genes are incorporated through plasmids, transposons, or integrons [14,16]. Adaptive resistance, often linked to biofilm formation, emerges during treatment, where biofilms limit antibiotic penetration and harbor persister cells—variants highly tolerant to antibiotics. These factors contribute to the chronicity and recurrence of *P. aeruginosa* infections [15].

The bacterium's pathogenicity is driven by diverse virulence factors that facilitate colonization, immune evasion, and tissue damage. Cell-associated components like flagella, pili, and lipopolysaccharides aid motility and adherence; while secreted factors such as enzymes, toxins, and pigments play central roles in tissue degradation and immune suppression [13]. The type III secretion system (T3SS), for instance, injects effector proteins exoenzyme S (*exoS*), exoenzyme T (*exoT*), exoenzyme U (*exoU*), and exoenzyme Y (*exoY*) into host cells, disrupting cellular processes and enhancing bacterial invasion. Biofilms, regulated by quorum sensing, not only shield bacteria, but also coordinate the expression of virulence factors, amplifying their pathogenic potential [2].

Pigments such as pyocyanin and pyoverdine further enhance virulence. Pyocyanin generates reactive oxygen species, causing oxidative tissue damage, particularly in CF lungs. Pyoverdine, a siderophore, enables iron acquisition that is essential for growth and infection establishment [17]. The combination of antimicrobial resistance and virulence, especially in multidrug-resistant (MDR) strains, poses a formidable clinical challenge, as antibiotic pressures may select for strains with heightened virulence.

Linking genotype to phenotype in *P. aeruginosa* is uniquely valuable in understanding its pathogenic mechanisms, including biofilm formation and toxin production. This approach reveals the molecular basis of resistance and virulence, providing insights into clinical outcomes and guiding the development of novel therapeutics [18]. Anti-virulence strategies targeting biofilms or specific toxins offer promise in mitigating infections without promoting resistance [19,20].

This study aimed to investigate the prevalence of phenotypic and genotypic virulence determinants in *P. aeruginosa*, and their associations with antibiotic resistance patterns in both MDR and non-MDR isolates. By correlating phenotypic markers, such as biofilm-forming capacity, with genotypic markers, this work provides novel insights into the relationship between resistance and virulence; thereby offering a comprehensive analysis to inform therapeutic strategies.

Methodology

In this cross-sectional study, *P. aeruginosa* was isolated from various clinical samples obtained from patients admitted to the Zagazig University Hospitals, Sharqia, Egypt; during the study period from June 2023 to June 2024. One hundred isolates were included in this study and they were recovered from urine (39), wound (32), sputum (21), and blood (8). The study was approved by the Zagazig Medical Research Ethical Committee (ZU-IRB#310/2- June 2024).

The isolates were cultivated on blood, MacConkey, and Mueller-Hinton agar media; incubated overnight at 37 °C; and then the non-lactose fermenting colonies and/or pigment producers were identified as *P. aeruginosa* using traditional available methods such as Gram stain, catalase test, oxidase test, growth at 42 °C, biochemical tests (motility indole ornithine (MIO), triple sugar iron (TSI), citrate, and urease agars), and the analytical profile index (API 20 E; bioMérieux , Marcy-L'Etoile, France) was used as a confirmatory test for the identification of *P. aeruginosa* isolates. The bacterial isolates were preserved in tryptic soy broth (TSB) containing 20% glycerol at – 70 °C [21], until further testing.

Antibiotic susceptibility tests

The susceptibility profiles of *P. aeruginosa* isolates were determined using the standard Kirby–Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) protocols [22]. The Muller-Hinton agar plates were inoculated with 0.5 McFarland suspensions of each isolate and incubated at 37 °C overnight. The isolates were tested against the following 13 antimicrobials discs, including 8 antibiotic classes: aztreonam (30 µg), amikacin (30 µg), ceftazidime (30 µg), cefepime (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), imipenem (10 µg), meropenem (10 µg), norfloxacin (5 µg), piperacillin (100 µg), piperacillin/tazobactam (100/10 µg), tetracycline (75 µg), and tobramycin (10 µg) (Oxoid, Basingstoke, UK). The results were interpreted as susceptible, intermediate, or resistant according to the CLSI recommendations [19]. For analytical purposes, isolates with intermediate and resistant results were grouped and reported as resistant. *P. aeruginosa* (ATTC 27853) was used as a quality control strain for the antimicrobial discs. The MDR isolates were identified if they showed resistance to at least 1 agent from 3 or more different antibiotic classes [23].

Phenotypic detection of some virulence factors

DNase production: Detection of DNase activity was investigated by spot inoculation of DNase test agar plates (Oxoid, Basingstoke, UK) by a colony of each isolate, incubation for 24 hours at 37 °C, followed by immersion of the surfaces of the plates with 1.0 N HCl. If a clear halo developed around the bacterial colony, the test was positive; while the lack of a clear halo denoted a negative test for DNase production [24].

Lipase production: Detection of lipase activity was determined by spot inoculation of Tween 80 agar plates with a single colony of each isolate, and incubation for 24 hours at 37 °C. After incubation, an opaque halo around the inocula indicated a positive test for lipase production; and absence of the halo indicated a negative test [25].

Pigment production: Pigment production was determined by visual evaluation for any pigment production in cetrimide agar. The plates were inoculated with the clinical isolate and incubated at 37 °C for 24 hours. The colonies were inspected for any pigments, including pyoverdine (green or yellow color with fluorescence upon exposure to ultraviolet light), pyocyanin (dark green- blue color), pyomelanin (brown color), or pyorubin (red color) [26].

Hemolysin production: To determine whether the isolates produced hemolysin, colonies of *P. aeruginosa* isolates were inoculated on 5% blood agar plates and incubated for 24 hours at 37 °C. Next, the plates were examined for the type of zone of hemolysis around the colonies based on the following criteria: β-hemolysis (clear zone), α-hemolysis (greenish zones), or γ-hemolysis (no hemolysis) [26].

Biofilm formation: The ability of MDR and non-MDR *P. aeruginosa* to form biofilm was assessed using a colorimetric tissue culture plate (TCP) method. The procedure described by Hassan *et al*. [26] was used to quantify the formation of biofilms, with some modifications. One colony, of every isolate, derived from fresh culture on Mueller Hinton agar was grown overnight at 37 °C in TSB containing 1% glucose. The bacterial suspensions were diluted 1:100 in a fresh TSB medium, and 200 µL of this solution was used to inoculate the sterile, polystyrene microtiter plates (96-well, flat-bottomed). Following a 48-hour incubation period at 37 °C, without agitation, the wells were washed gently 3 times with distilled water. Next, the plates were inverted and air dried. The adhering bacteria were fixed for 10 minutes in absolute methanol, before being stained with 200 µL of 0.1% crystal violet (CV) solution for approximately 20 minutes. The CV was discarded, and the wells were triple-washed to get rid of any remaining CV. PA PAO1 and TSB medium were used as positive and negative controls. Finally, 200 µL of 30% acetic acid was added to dissolve the bounded CV. An enzyme-linked immunosorbent assay (ELISA) reader was used to measure the absorbance of optical density (OD) of each well at 550 nm. Every assay was conducted 3 times. The uninoculated medium was used as a control to measure background OD. The cutoff OD (ODc) was defined as 3 standard deviations above the mean OD of the negative control. Following the microtiter plate test, the isolates were categorized into 4 groups based on OD: those that did not produce biofilm (OD test < ODc), those that produced weak biofilm (ODc < OD > 2x ODc), those that produced moderate biofilm (2x ODc < OD > 4x ODc), and those that produced strong biofilm (4x ODc < OD).

The selection of specific virulence factors; such as DNase, lipase, pigment production, hemolysin, and biofilm formation; is crucial as these factors significantly contribute to the pathogenicity, survival, and adaptability of *P. aeruginosa* in various host environments. Investigating these factors provides insights into the mechanisms underlying infection severity and potential therapeutic targets for combating MDR strains.

Molecular detection of genes encoding virulence factors

Genomic DNA extraction was performed using the boiling method. Six colonies from each cultured and confirmed *P. aeruginosa* plate were dissolved in 100 µL of DNase/RNase-free water in sterile Eppendorf tubes. The suspension was lysed by placing it in a boiling water bath at 100 °C for 10 minutes, which facilitated the preparation of the DNA template. After this, the samples were centrifuged at 10,000 rpm for 10 minutes, and the supernatant was collected into another sterile tube. The extracted DNA was then stored at − 20 °C for further use [19].

Polymerase chain reaction (PCR) amplification was carried out using previously published primers and a thermal cycler (Applied Biosystems, Foster City, CA, USA). The reactions were prepared in a 25 µL volume. The PCR conditions were optimized for each target gene. These genes were selected for their roles in biofilm formation, hemolysin activity, toxin secretion, and other resistance mechanisms.

For *toxA*, *plcH*, and *algD*; the protocol consisted of an initial denaturation step at 94 °C for 3 minutes. This was followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 60 seconds, and

extension at 72 °C for 90 seconds; with a final extension at 72 °C for 5 minutes.

For *lasB* and *aprA*, the protocol included a preliminary denaturation at 94 °C for 4 minutes; followed by 25 cycles of denaturation at 94 °C for 60 seconds, annealing at 46 °C for 40 seconds, and extension at 72 °C for 60 seconds; and a final extension at 72 °C for 2 minutes.

The amplification protocol of *exoS* and *exoU* included an initial denaturation at 95 °C for 5 minutes; followed by 35 cycles of denaturation at 95 °C for 60 seconds, annealing at 55 °C for 60 seconds, and extension at 72°C for 60 seconds; with a final extension at 72 °C for 10 minutes [27].

Each gene was amplified independently, and the resulting PCR products were analyzed using gel electrophoresis. The amplified DNA fragments were separated on a 1% agarose gel in a tris acetate EDTA buffer; run for 60 minutes at 100 V. The gel was then stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) to visualize the DNA bands. The stained bands were observed under UV light and compared against a DNA ladder (iNtRON Biotechnology, Seongnam, South Korea) to determine the amplicon sizes.

The sequences of the primers used for PCR amplification of each target gene and their corresponding amplicon sizes are presented in Table 1.

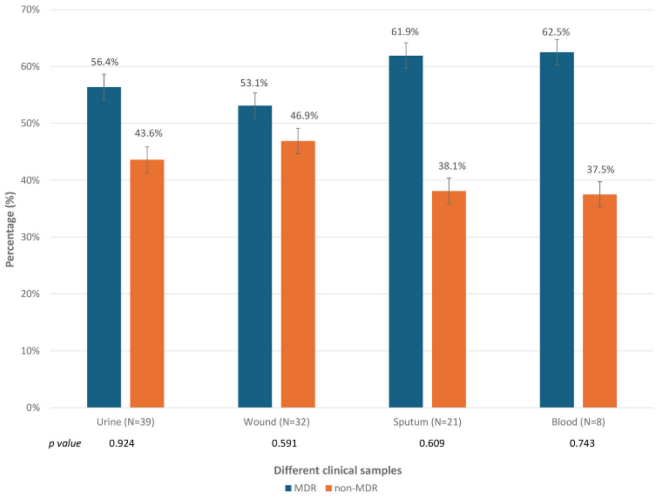
Statistical analysis

The data collected were analyzed using IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA). The categorical variables (qualitative data) were expressed as percentages and counts. The Chi-square test was used for comparisons, and *p* values less than 0.05 were considered significant.

Results

In this study, 100 *P. aeruginosa* clinical isolates were isolated from urine (n = 39), wound (n = 32), sputum (n = 21), and blood cultures (n = 8). They displayed 27 different susceptibility patterns (13 non-MDR and 14 MDR) and were classified into 2 categories: non-MDR (43%) that were resistant to < 3 antibiotic classes, and MDR (57%) that were resistant to ≥ 3 antibiotics classes [20]. Among all isolates, 62.5% were recovered from bloodstream samples, 61.9% from sputum, 56.4% from urine, and 53.1% from wound cultures respectively. However, there was no statistically significant association between non-MDR and MDR isolates, and the sample type (Figure 1).

Figure 1. Distribution of non-MDR and MDR *P. aeruginosa* in different clinical samples.



MDR: multidrug resistant.

The highest resistance rate among the isolates was detected for ceftazidime (63%), followed by cefepime and tobramycin (62% for each). Aztreonam resistance (94.7%) was the highest among MDR isolates, followed by piperacillin (89.5%), tobramycin (89.5%), and cefepime (87.7%). Among the non-MDR isolates the highest resistances were to ceftazidime (44.2%) and ciprofloxacin (41.9%). On the other hand, the isolates were the most susceptible to carbapenems (meropenem and imipenem) in both categories (Figure 2).

The frequencies of virulence factors among the isolates were as follows: pigment production was observed in 94% of isolates, with pyocyanin (51%) and pyoverdine (43%). Biofilm formation was detected in 93% of isolates, categorized as strong (45%), moderate (28%), and weak (20%). The prevalence of specific virulence genes and enzymes was as follows: *algD* (91%), *lasB* (90%), *toxA* (86%), *exoS* (82%), *aprA*

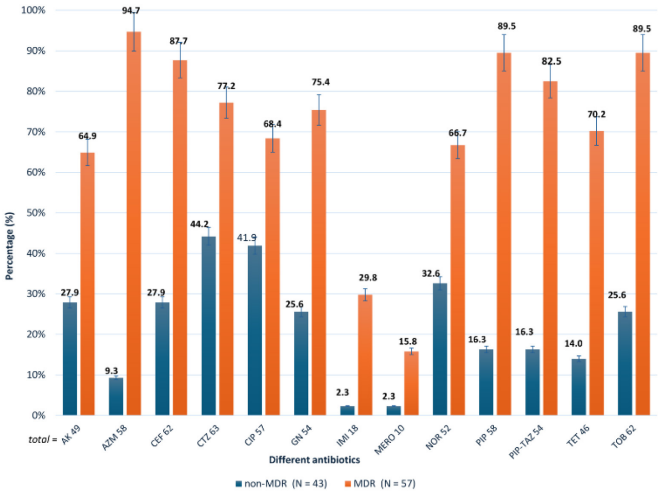
Table 1. Primer sequences and amplicon sizes for the detection of virulent genes in *P. aeruginosa.*

Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Amplicon Size (bp)
ToxA	GGTAACCAGCTCAGCCACAT	TGATGTCCAGGTCATGCTTC	352
PlcH	GAAGCCATGGGCTACTTCAA	AGAGTGACGAGGAGCGGTAG	307
algD	ATGCGAATCAGCATCTTT	CTACCAGCAGATGCCCTCGGC	1310
LasB	CCAGCCCGCTGACCCACAAGCTGTA	CATTCCTTCCTGGAGTGCYRGCCG	665
AprA	CCTGATCKGGCCGATAACTGCAAT	GGAAGACASCTATCAATTCGAACAG	1609
ExoU	GGCACATATCTCCGGTTCCTTC	TCAACTCAGCTGCCAACCATGC	761
ExoS	ATGGCGTGTTCCGAGTCA	AGGTGTCGGTTCGTGACGTCT	1587

(78%), *plcH* (75%), hemolysin (72%), lipase (65%), DNase (36%), and *exoU* (19%). The most and the least frequent genotypic virulence factors among MDR isolates were *algD* (91.2%) and *exoU* (19.3%); and pigment production (96.5%) was the most frequently detected phenotypic virulence factor; of these 50.9% and 45.6% were pyocyanin and pyoverdine respectively. On the other side, DNase was the least detected phenotypic factor (28.1%). Among non-MDR isolates, *lasB* (93.0%) and *exoU* (18.6%) were the most and the least frequent genotypic virulence factors; while biofilm formation (93.0%) and DNase (46.5%) were the most and the least frequent phenotypic factors. There was no significant association of any of the studied virulence factors with either MDR or non-MDR isolates, except for strong biofilm formation that was significantly more associated with MDR isolates (p = 0.01) (Figure 3).

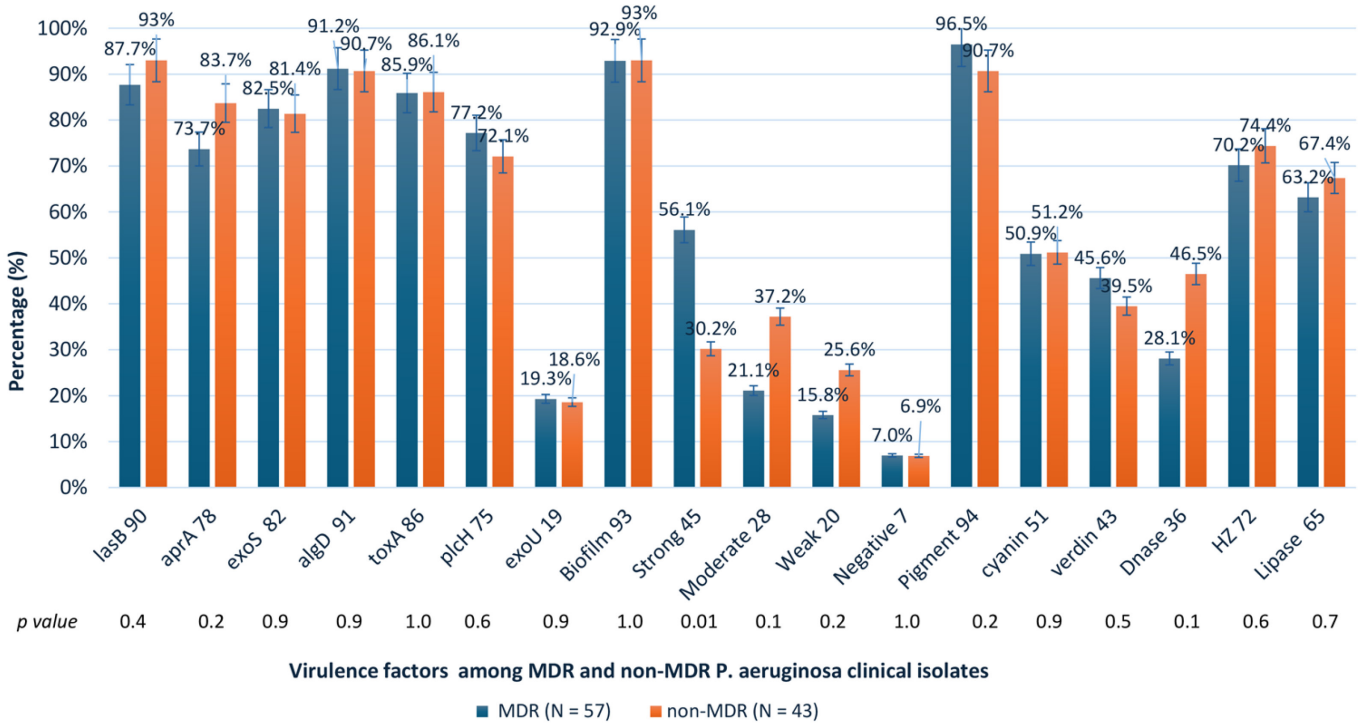
The associations between virulence factors and antibiotic susceptibility or resistance among the isolates were as follows: *aprA* was more associated with the strains susceptible to aztreonam, piperacillin, and piperacillin\tazobactam; *exoS* was associated with tobramycin susceptibility; pigment production was associated with imipenem susceptibility; DNase was associated with aztreonam and norfloxacin susceptibility; and lipase was associated with tobramycin and ceftazidime susceptibility. Strong biofilm was more associated with strains that were resistant to aztreonam, cefepime, ceftazidime, piperacillin, and tobramycin. *lasB*, *algD*, *toxA*, *plcH*, *exoU*, biofilm production except strong biofilm, and

Figure 2. Distribution of antibiotic resistance patterns in MDR and non MDR *P. aeruginosa* clinical isolates.



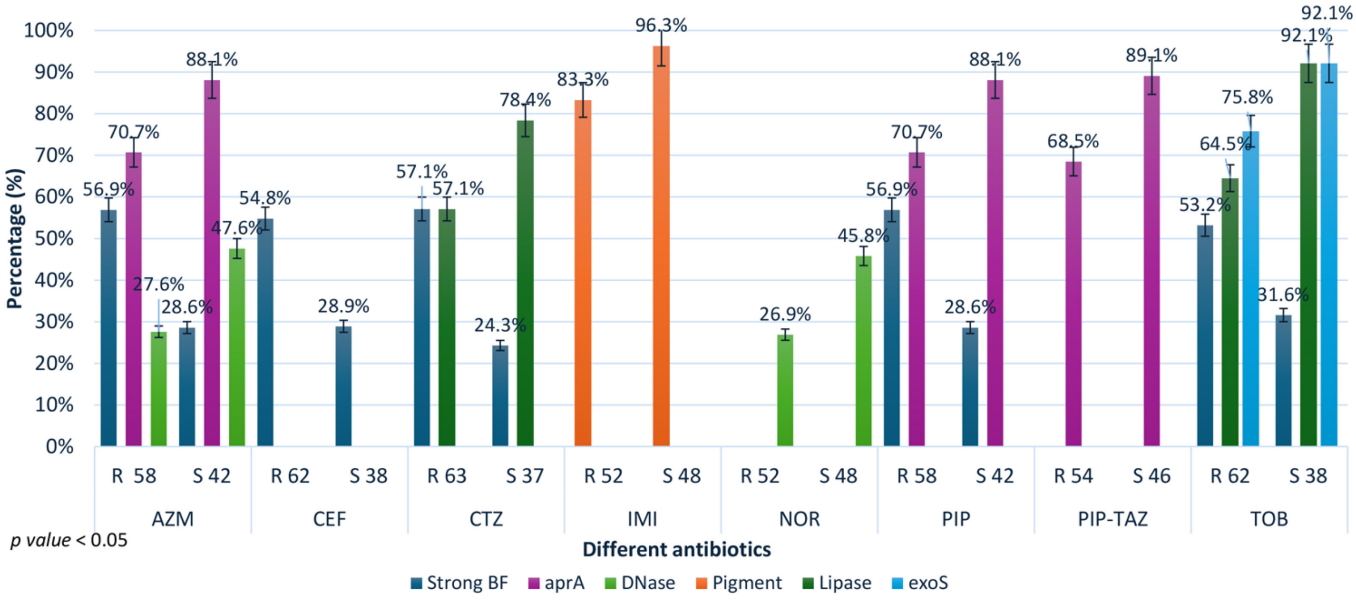
AK: amikacin; AZM: aztreonam; CEF: cefepime; CTZ: ceftazidime; CIP: ciprofloxacin; GN: gentamicin; IMI: imipenem; MERO: meropenem; NOR: norfloxacin; PIP-TAZ: piperacillin/tazobactam; PIP: piperacillin; TET: tetracycline; TOB: tobramycin; MDR: multidrug resistant.

Figure 3. Frequency of virulence factors among MDR and non-MDR *P. aeruginosa* clinical isolates.



Hz: hemolysin.

Figure 4. Distribution of virulence factors among susceptible and resistant *P. aeruginosa* strains.



AZM: aztreonam; CEF: cefepime; CTZ: ceftazidime; IMI: imipenem; NOR: norfloxacin; PIP-TAZ: piperacillin/tazobactam; PIP: piperacillin; TOB: tobramycin; BF: biofilm; R: resistant; S: sensitive.

hemolysin had no significant association with either susceptible or resistant strains. In addition, all the studied virulence factors had no significant association with either susceptible or resistant strains tested against amikacin, ciprofloxacin, meropenem, and tetracycline (Figure 4.).

Biofilm formation and pigment production were strongly correlated with their respective molecular factors in both MDR and non-MDR isolates. Strong biofilm formation showed a significant correlation with MDR isolates, indicating its potential role in resistance (Table 2).

All studied isolates from different clinical specimens had at least 4 virulence factors. 57% of the isolates harbored 4–5 genes, and 61% of the isolates were positive for 4–5 virulence factors. No significant difference was noted between MDR and non-MDR isolates regarding the distribution of virulence factors ($p > 0.05$) (Figure 5).

The *exoS* and *exoU* were detected in 5 isolates; 3 MDR isolates (1 each from blood, sputum, and urine), and 2 non-MDR isolates (from sputum; Supplementary Figure 1).

Discussion

P. aeruginosa is a highly adaptable opportunistic pathogen, notorious for its association with increased morbidity and mortality in healthcare settings. The emergence of MDR strains has intensified this challenge, prompting the World Health Organization (WHO) to designate *P. aeruginosa* as a "critical" pathogen, highlighting the urgent need for new antibiotics [27,28]. In our study, 57% of the tested isolates were MDR, aligning with various international reports, although some studies have indicated even higher MDR rates [29–34]. This significant MDR prevalence underscores the urgent need for stringent antibiotic prescription guidelines [29].

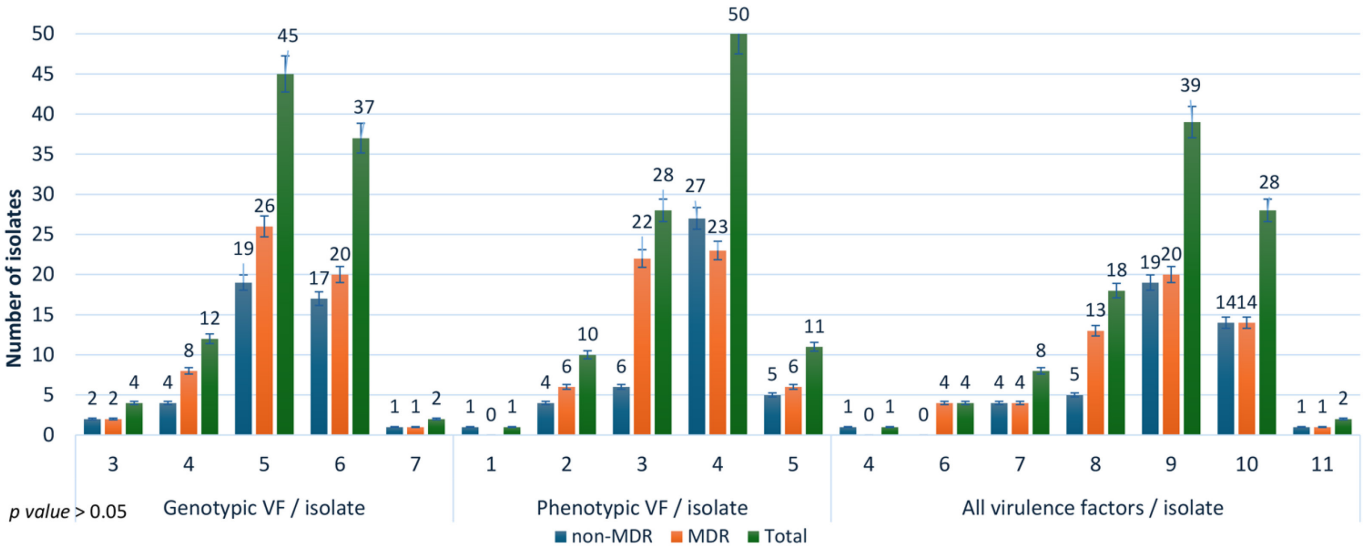
Among the antibiotics tested, ceftazidime exhibited the highest resistance rate at 63%, a finding consistent with several studies [35,36]. Even more concerning was the resistance to aztreonam, with 94.7% of MDR isolates displaying resistance; a trend similarly observed in studies from Gaza and Iran [36,37]. In contrast, carbapenems such as meropenem and imipenem showed the lowest resistance rates; with 2.3% for both in non-MDR isolates, and 15.8% and

Table 2. Correlation between phenotypic and molecular virulent factors.

Phenotypic virulent factor	Associated gene (s)	MDR (n = 57)	Non-MDR (n = 43)	Correlation observed
Biofilm formation	*algD, lasB*	53 (93.0%)	40 (93.0%)	Strong correlation with *algD* and *lasB*
Pigment production	*toxA, lasB*	55 (96.5%)	39 (90.7%)	Correlation with *toxA* and *lasB*
Hemolysin activity	*plcH*	40 (70.2%)	32 (74.4%)	Moderate correlation
Lipase production	*aprA*	36 (63.2%)	29 (67.4%)	Weak correlation with *aprA*
Strong biofilm	*algD*	32 (56.1%)	13 (30.2%)	Significant correlation

MDR: multi-drug resistant.

Figure 5. Distribution of genotypic, phenotypic, and all virulence factors in the MDR and non-MDR isolates of *P. aeruginosa*.



VF: virulence factor.

29.8% in MDR isolates, respectively; consistent with other studies reporting low carbapenem resistance [37,38].

The genome of *P. aeruginosa* is equipped with a wide array of virulent factors that significantly contribute to its pathogenicity. These factors endow the bacterium with remarkable metabolic flexibility and adaptability to various conditions, including evading the host immune response [39]. Among these factors, pigment production, a cytotoxic feature contributing to its pathogenesis, was observed in 94% of the tested isolates. This result is comparable to previous studies, such as one from Egypt, which reported an 84.6% incidence of pigment production [29]. Pyocyanin and pyoverdine were the two pigments detected, with pyocyanin being slightly more prevalent. While no significant difference in pigment production was observed between MDR and non-MDR isolates, pyocyanin was more associated with imipenem susceptibility [29,30]; although other studies have linked it to ceftazidime susceptibility [40].

Hemolysin, another crucial virulence factor, was detected in 72% of the isolates, with no significant difference between MDR and non-MDR strains. This aligns with a previous study that reported similar rates [30], although other studies have reported higher rates of hemolysin production [29].

DNase, which aids in biofilm matrix maintenance and immune evasion, was detected in 36% of the tested isolates, making it the least common virulence factor observed. This aligns with studies reporting DNase detection rates ranging from 2.0% to 72.2% [41]. DNase production showed no significant difference between MDR and non-MDR isolates, but was more associated with susceptibility to aztreonam and norfloxacin. Similar associations with antibiotic susceptibility, particularly with intermediate resistance to meropenem, have been reported by others [29].

Lipase, an extracellular enzyme contributing to drug resistance by interacting with alginate in the biofilm matrix, was found in 65% of the tested isolates. Lipase production was slightly higher in non-MDR isolates, though the difference was not statistically significant. This finding is consistent with studies reporting similar frequencies of lipase production, ranging from 54% to 100% [42,43]. Moreover, lipase production was more associated with strains susceptible to tobramycin and ceftazidime, further emphasizing its role in antibiotic susceptibility [44].

Biofilm formation, one of the most critical pathogenic factors in *P. aeruginosa* infections, was observed in 93% of the studied isolates, with 45% identified as strong biofilm producers. Although no significant differences between MDR and non-MDR groups were noted, strong biofilm production was significantly higher in MDR isolates (56.1%). This finding is consistent with other studies reporting a significant association between strong biofilm production and MDR status [45,46]. The strong biofilm production observed in MDR isolates (56.1%) likely contributes to therapeutic failures by impeding antibiotic penetration. These findings align with studies from Asia and the Middle East, emphasizing the global challenge of MDR *P. aeruginosa* [47]. Strong biofilm

producers in our study were also more likely to be resistant to aztreonam, cefepime, ceftazidime, piperacillin, and tobramycin; whereas a study in Turkey found no significant relationship between biofilm formation and antibiotic resistance. Incorporating biofilm-targeting agents into treatment regimens could significantly improve outcomes for MDR *P. aeruginosa* infections [48]

The *algD* gene, crucial for biofilm maturation and bacterial persistence, was detected in 91% of the tested isolates. This observation aligns with studies reporting the high prevalence of the *algD* gene without a direct link to biofilm formation [49]. However, in our study, the presence of the *algD* gene did not significantly differ between MDR and non-MDR isolates, nor did it correlate with biofilm density. This observation is consistent with other studies that have reported a high prevalence of the *algD* gene without a direct link to biofilm formation [38,50]. Notably, *algD* was present in both biofilm and non-biofilm producers, suggesting a more complex role in biofilm formation than previously understood [51].

Our study also examined the prevalence of the *lasB* and *toxA* genes, that encode vital extracellular toxins during infection. *lasB* and *toxA* were present in 90% and 86% of the isolates, respectively, with no significant difference between MDR and non-MDR strains. This finding aligns with studies reporting high prevalence rates for these genes, though some have noted associations between *toxA* and ceftazidime susceptibility [38,52]. The variability in *lasB* across strains highlights the genetic diversity of *P. aeruginosa* and its adaptability [53].

The *aprA* gene, responsible for producing alkaline protease, was detected in 78% of the strains, with no significant difference between MDR and non-MDR isolates. Similar findings have been reported in other studies [34]. However, *aprA* was more associated with susceptibility to aztreonam, piperacillin, and piperacillin/tazobactam; similar findings have been reported in other studies [54].

The *hemolytic phospholipase C* (*plcH*) gene, linked to organ damage and cell death, was present in 75% of the isolates. While there was no significant difference between MDR and non-MDR strains in our study, other studies have reported varying *plcH* prevalence rates, ranging from 38.8% to 96.1% [55–57]. Some studies have found a significant association between *plcH* and aztreonam resistance, while others have linked it to piperacillin-tazobactam susceptibility [29,31].

Exotoxins U and S, encoded by the *exoU* and *exoS* genes, are among the most virulent effectors in *P. aeruginosa* infections. *exoU*, a potent phospholipase, was present in 19% of the isolates, consistent with previous findings [58]. The presence of *exoU* showed no significant difference between MDR and non-MDR strains; though some studies have reported a stronger association with MDR status [59]. The *exoS* gene was detected in 82% of the isolates, and was more associated with tobramycin susceptibility in our study; a finding also reported by others linking it to ceftazidime and aztreonam susceptibility [60]. Interestingly, while *exoU* and *exoS* genes rarely coexist in the same strain, 5 of our isolates (5%) carried both genes; a phenomenon also observed in other studies [26,40].

All the isolates that we tested expressed virulent genes, with only 2% expressing all the studied genes. About 57% of the isolates harbored 4–5 virulent genes, and 11% were positive for all 5 phenotypic tests. This pattern of virulence factor expression is similar to other studies which reported that 18.3% of isolates possessed all tested genes, and 58.7% harbored 4–5 genes [29]. Despite the high prevalence of virulence factors, our study found no significant correlation between MDR status and the expression of these virulence factors, except for strong biofilm production [59].

The finding that MDR isolates often carried multiple virulence factors, with some strains harboring as many as 11 different factors, is particularly concerning. This combination of high-level antibiotic resistance and extensive virulence factor repertoire suggests that these MDR strains are not only difficult to treat but also pose a significant risk for causing severe and persistent infections.

Conclusions

This study revealed that despite the fact that MDR in *P. aeruginosa* is becoming more common, carbapenems are still effective against it. Virulence factors were prevalent among the isolates, with pigment production and biofilm formation being the most common; and strong biofilm formation was significantly associated with MDR isolates. Additionally, certain virulence factors were linked to specific antibiotic susceptibility; but overall, there was no significant difference in the distribution of these factors between MDR and non-MDR isolates. These findings highlight the complexity of *P. aeruginosa* infections and the need for targeted therapeutic strategies to manage MDR strains effectively. Future research should focus on longitudinal studies to monitor the evolution of resistance and virulence, mechanistic studies to understand their associations, exploration of

alternative therapies, assessment of environmental factors, and clinical trials to evaluate combination therapies.

Acknowledgements
We would like to express our sincere gratitude to all individuals and institutions that contributed to this study. Special thanks to the staff at Zagazig University Hospitals for their assistance in collecting clinical isolate samples and providing access to laboratory facilities. We also extend our heartfelt appreciation to the Microbiology Department at Zagazig University for their valuable insights and support throughout the research process. Finally, we are deeply grateful to Al-Azhar University and Ain Shams University for their unwavering support and for fostering a collaborative environment that greatly enriched this study.

Authors' contributions
SAIA, concept development, methodology, data collection and analysis, manuscript preparation; AAG, HKM, AMS, data collection and analysis, manuscript preparation; AMA, concept development, design, methodology, data analysis, manuscript preparation and revision; NGE, editing, revising, and analyzing the manuscript, MMA, methodology development, data collection and analysis. All authors read and approved the final version of the manuscript.

Data availability
The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethical approval
Ethical approval was granted by the Zagazig Medical Research Ethical Committee (ZU-IRB#310/2-June-2024).

Corresponding author
Abdullah M Abdo, PhD.
Al-Azhar University, Faculty of Science, Botany and Microbiology Department,
Housing officers, the new El-Zawya Al-Hamra, Block 60, Entrance 2, Flat 3, Cairo, Egypt.
Tel: +201141801022
Fax: +20 2 24268311
Email: abdullah.abdo@azhar.edu.eg

Conflict of interests
No conflict of interests is declared.

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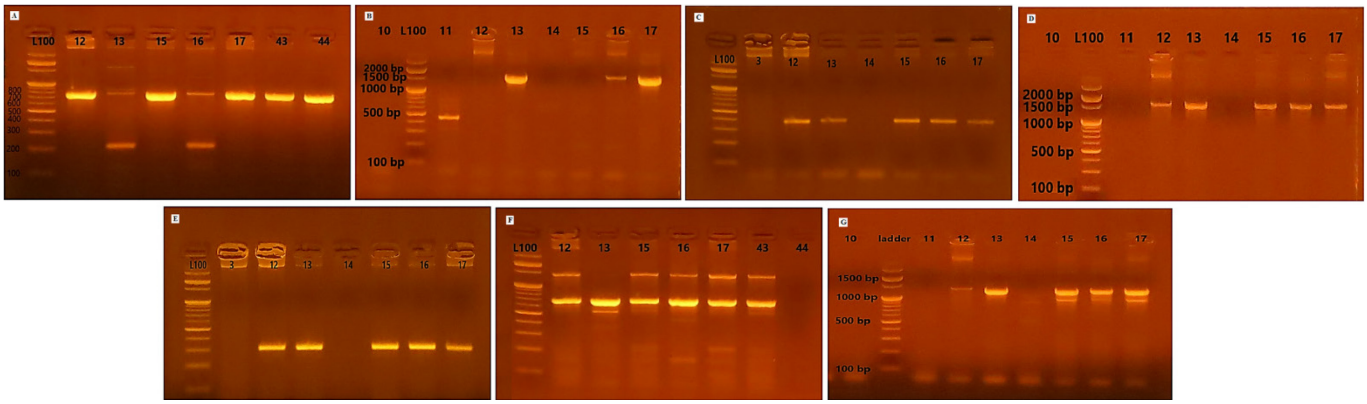
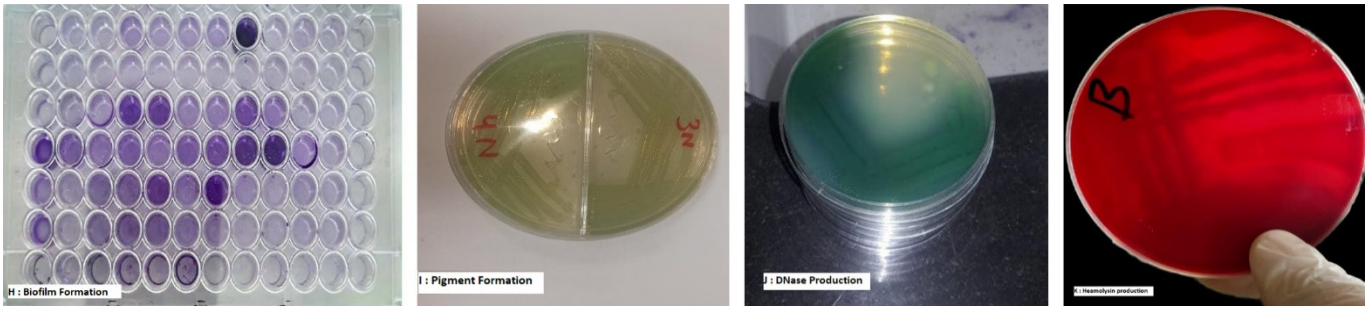
Annex – Supplementary Items

Supplementary Figure 1A–G. Detection of genotypic and phenotypic virulence factors in *P. aeruginosa*.



A: exoU gene (761 bp); B: exoS gene (1587 bp); C: toxA gene (352 bp); D: aprA gene (1609 bp); E: plcH gene (307bp); F: lasB gene (665bp); G: algD gene (1310bp).

Supplementary Figure 1H-K. Detection of genotypic and phenotypic virulence factors in *P. aeruginosa*.



H: biofilm formation; I: pigment formation; J: dNase production; K: hemolysin production.