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Plasmid profiles and antibiotic resistance of *Pseudomonas aeruginosa* isolated from different clinical samples in Jordan

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ABSTRACT

Aims: The aim of this study was to investigate plasmid profiles and antibiotic resistance patterns of multidrug resistant *Pseudomonas aeruginosa* strains isolated from different clinical specimens in Jordan.
Methodology and results: *Pseudomonas aeruginosa* strains were isolated from different clinical specimens from different hospitals and primary health care centers. The antimicrobial susceptibility of *P. aeruginosa* isolates was determined using the disc diffusion method against 16 commonly used antimicrobial drugs. Plasmid DNAs were extracted from lysed *P. aeruginosa* cells using the plasmid alkaline lysis method and visualized using agarose gel electrophoresis followed by ethidium bromide staining. The isolated strains of *P. aeruginosa* were resistant to cefepime (90%), meropenem (70%), ceftazidime (60%), piperacillin (55%), aztreonam (50%), ciprofloxacin and tobramycin (35%), gentamicin (29%), imipenem and amikacin (20%). All the isolates were sensitive to colistin. Plasmid analysis of the clinical isolates showed the presence of 0 to 3 plasmids with a size range of 1 to 25 kb compared to the standard strain of *Escherichia coli* (ATCC 25922).
Conclusion, significance and impact of study: The results obtained in this study showed some correlation between the patterns of antibiotic resistance and plasmid profiles.

Keywords: Antibiotic resistance, clinical isolates, DNA, plasmids, *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium with a widespread niche in many environments, including humans, plants and animals (Lister *et al*., 2009). It causes nosocomial infections, especially in immunocompromised individuals, such as hospitalized patients, infected people with the human immunodeficiency virus (HIV) and cancer patients (Gale *et al.*, 2015; Wu *et al.*, 2015; Gomila *et al.*, 2018). It was recognized in 2017 as one of the most dangerous and life-threatening bacteria that need intensive research and development of antibiotics by the World Health Organization (WHO) (Pang *et al.*, 2019). The ability of *P. aeruginosa* to form biofilms makes it resistant to different antimicrobial treatments and phagocytosis, which can lead to long-term colonization of the body (Moradali *et al.*, 2017). Biofilms were observed in patients with chronic infections such as lung, rhinosinusitis and chronic wound infections (Römling and Balsalobre, 2012). Diagnosing infections with *P. aeruginosa* at an early stage is important to ensure effective treatments and prevent its conversion to chronic infections.

Pseudomonas aeruginosa is usually not part of the indigenous microflora of humans and animals; the colonization rate can range between 0-2.6% for different anatomical sites (skin, nasal passages, throat and fecal samples (Lister *et al*., 2009). However, colonization increases to about 50% with compromised individuals (Vallés *et al.*, 2004; Lister *et al*., 2009).

Several mechanisms of resistance to antimicrobial agents have been described in clinical isolates of *P. aeruginosa*, ranging from efflux pump to mobile genetic elements (plasmids), as well as hydrolyzing enzymes (Mesaros *et al*., 2007, Odumosu *et al*., 2013; Odumosu *et al*., 2015a; 2015b). *Pseudomonas aeruginosa* accounts for up to 10% of all human infections and is one of the important bacterial pathogens commonly isolated from various clinical and environmental samples (Rajat *et al*., 2012).

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The genes conferring resistance in *P. aeruginosa* are located on transferable genetic elements such as plasmids, transposons, integrin class 1 or on the bacterial chromosome; these genetic elements can be transferred to other Gram-negative bacteria contributing to the spread of antimicrobial resistance in hospitalized patients and the general population (Giedraitienė *et al*., 2011). It has been reported that hospital-associated (nosocomial) and community-acquired *P. aeruginosa* infections may be resistant to many antimicrobial compounds, including antiseptics and disinfectants (Massadeh and Jaran, 2009a; 2009b). This resistance may result, in part, from the acquisition of new genetic material either by mutations within chromosomal genes, through plasmids or via transposable genetic elements, such as transposons (Odumosu *et al*., 2015a; 2015b). Plasmids are double-stranded small circular DNA molecules; they can self-replicate within the bacteria. Plasmids do not usually carry genes essential for the growth of bacterial cells, but the genes they carry could be expressed under stress conditions (Rajat *et al*., 2012). Several studies carried out to determine the role of plasmids on antibiotic resistance have been useful in determining the characteristics of plasmids in bacteria (Fatima *et al*., 2012). Plasmids can be transferred horizontally between close bacteria via pili, while plasmids can be transferred phylogenetically for bacteria that are distant from one another (Breidenstein *et al*., 2011).

The objectives of the present study were to investigate the antibiotic resistance patterns and plasmid profiles of antibiotic-resistant *P. aeruginosa* strains isolated from different clinical sources.

MATERIALS AND METHODS

Specimen collection

Fifty non-repetitive *P. aeruginosa* samples were collected from different clinical sources (urine, pus, wounds, burns and sputum). Ten of each of the specimens were collected aseptically from patients attending different hospitals and primary health care centers in northern Jordan from September 2019 to March 2020. This study was conducted at the Department of Medical Laboratory Science, Faculty of Science, Al al-Bayt University, Mafraq, Jordan. Approval for this study was obtained from the ethical committee at Al al-Bayt University, Jordan.

All isolates were identified using routine laboratory procedures: Direct microscopic examination using Gram stain, basic colonial morphology, cultural characteristics on *Pseudomonas* media, pigment production (green coloration), catalase test, oxidase tests and slide coagulase test. Isolates that were Gram-negative rods, positive to catalase and oxidase tests, and slide coagulase test were considered as *P. aeruginosa*.

Antimicrobial susceptibility

Antimicrobial susceptibility of *P. aeruginosa* isolates was determined using Kirby-Bauer disc diffusion method (Bauer *et al*., 1966) against 11 commonly used antimicrobial drugs, namely amikacin (AK) (30 µg), aztreonam (ATM) (30 µg), ciprofloxacin (CIP) (5 µg), ceftazidime (CAZ) (30 µg), tobramycin (TOB) (30 µg), meropenem (MEM) (10 µg), piperacillin (PIP) (100 µg), gentamicin (GM) (10 µg), imipenem (IMI) (10 µg), colistin (CT) (10 µg) and cefepime (FEP) (30 µg) (Abtek, U.K.). The test was performed on Mueller Hinton agar plates according to the guidelines of the Clinical and Laboratory Standards Institute, CLSI (2019) (Bauer *et al*., 1966).

Pseudomonas aeruginosa was grown in Mueller-Hinton broth overnight and then adjusted to the turbidity of 0.5 McFarland standard. Bacteria were cultured on Muller-Hinton agar plates before antibiotic discs were placed on the surface of the agar, and the plates were incubated at 37 °C for 18 h. Strains of *E. coli* (ATCC 25922) were used in the study as a control.

The size of the area of suppressed growth (zone of inhibition) was determined by the concentration of the antibiotics present in the area and, therefore, the diameter of the inhibition zone represents the relative susceptibility to a particular antibiotic. The interpretation of the results as sensitive or resistant was determined according to standard charts provided by the manufacturer (OXOID Limited, Basingstoke, Hampshire, England).

Plasmid isolation

The selected *Pseudomonas* bacterial strain (as a single colony) was grown overnight in Luria-Bertani (LB) broth at 37 °C with aeration using an orbital shaker. Plasmid DNA was extracted from lysed bacterial cells using alkaline lysis according to the method of Takahashi and Nagano (Takahashi and Nagano, 1984).

Agarose gel electrophoresis of plasmid DNA

Plasmid DNA bands were detected by electrophoresis on a 0.8% horizontal agarose gel (Fisher Biotech, New Jersey, USA) pre-stained with ethidium bromide (0.5 µg/mL) and visualized under UV light. The approximate molecular weight of plasmids (in mega Daltons) was determined by extrapolation based on the mobility of Hind III digested λ DNA (Promega-USA) co-electrophoresed with the plasmid DNA samples (Meyers *et al*., 1976; Jamieson et al., 1979; Litwin *et al*., 1991).

Statistical analysis

The correlation of plasmids to antibiotic resistance was calculated using Microsoft Excel (2016) program.

RESULTS AND DISCUSSION

Pseudomonas aeruginosa is one of the most dangerous bacteria of the century, affecting a broad range of people with different health problems including pneumonia, cystic fibrosis, chronic obstructive pulmonary disease, HIV and cancer patients (Hancock and Speert, 2000; Gale *et al.*, 2015; Wu *et al.*, 2015; Daury *et al*., 2016; Gomila *et al.*,

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2018; Udea *et al*., 2021). It is one of the pathogens that needs urgent research and development of new antibiotics, according to the WHO (Munita and Arias, 2016; Thacharodi and Lamont, 2022).

Antimicrobial resistance can be the result of one or more of four main categories: limiting the uptake of a drug, modifying a drug target, inactivating a drug and active drug efflux. These mechanisms can be native to the microorganism itself or acquired by bacteria from other microorganisms. The resistance mechanisms vary between Gram-negative and Gram-positive bacteria due to differences in their cell wall structure. Gram-negative bacteria use all four main mechanisms stated above, whereas Gram-positive bacteria, due to their lack of LPS outer membrane, less commonly can use the mechanism of limiting the uptake of a drug and do not have the capacity for certain types of drug efflux mechanisms (Chancey *et al*., 2012; Mahon *et al*., 2014).

We studied different antibiotic resistance to *P. aeruginosa* strains isolated from different clinical samples (urine, pus, wounds, burns and sputum). The bacteria were first tested using the Gram stain technique (Figure 1) as well as other different tests before the study to confirm the presence of *P. aeruginisa*. Our results showed that two isolates were resistant to six antibiotics, four were resistant to five antibiotics, and five were resistant to four antibiotics. Isolates resistant to three antibiotics accounted for 48%, isolates resistant to two antibiotics accounted for 21%, isolates resistant to one antibiotic accounted for 18% and 12% of the total isolates were sensitive to all antibiotics tested. Our results showed that the clinical isolates had varying patterns of resistance to the antibiotics tested with high resistance to cefepime (90% resistance), meropenem (70%), ceftazidime (60%) and piperacillin (55%); moderate resistance to aztreonam (50%), ciprofloxacin and tobramycin (35%), gentamicin (29%), imipenem and amikacin (20%) (Figure 2). All the strains were sensitive to colistin. Table 1 represents the pattern of distribution of all *P. aeruginosa* isolated strains in this study depending on their clinical source. Our findings are similar to other studies on *P. aeruginosa*, showing similar patterns of antibiotic resistance to quinolones, aminoglycosides and β-lactams (Hancock and Speert, 2000). Multidrug-resistant (MDR) *P. aeruginosa* that showed resistance to three or more different classes of antibiotics have been identified in this study, and the isolated strains displayed an MDR phenotype against several antibiotics: cefepime (90%), meropenem (70%) and ceftazidime (60%). MDR of *P. aeruginosa* was influenced by different factors, including a decrease in the bacteria's outer membrane permeability, leading to the expulsion of antibiotics (Udea *et al*., 2021). The expression of 4 major efflux pumps (MexAB-OprM, MexXY, MexCD-OprJ and MexEF-OprN) was found to be linked to the MDR of *P. aeruginosa* (Daury *et al*., 2016); these efflux pumps are transmembrane protein complexes that work as drug/proton antiporters catalyzing the extrusion of their specific substrates including from the periplasm through the outer membrane (Venter *et al*., 2015). Henrichfreise *et al.* (2007) showed that in addition to the high level of intrinsic antibiotic resistance of *P. aeruginosa*, the acquired resistance greatly contributes to the development of multidrug-resistant strains, which

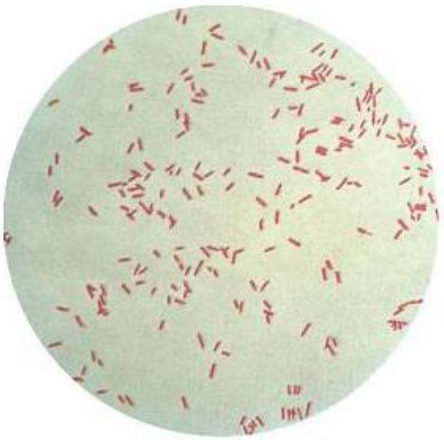

Figure 1: Image of the Gram-negative *Psedomonas aeruginosa* following staining by the Gram stain technique.

Table 1: Pattern of antibiotic resistance to all *Pseudomonas aeruginosa* strains isolated from different clinical sources (urine, pus, wounds, burns and sputum). The table indicates the number of *P. aeruginosa* isolates and the corresponding clinical source.

Antibiotics	Number of resistant isolates	Clinical source
Cefepime	45	All specimens
Meropenem	35	Urine, pus, wound and burn
Ceftazidime	25	Urine, pus and wound
Piperacillin	28	Urine, pus, burn and sputum
Aztreonam	25	Urine, wound and sputum
Ciprofloxacin	18	Urine, pus and burn
Tobramycin	18	Urine and pus
Gentamicin	14	Pus, wound and burn
Amikacin	10	Pus, wound and burn
Imipenem	10	All specimens
Colistin	0	All specimens

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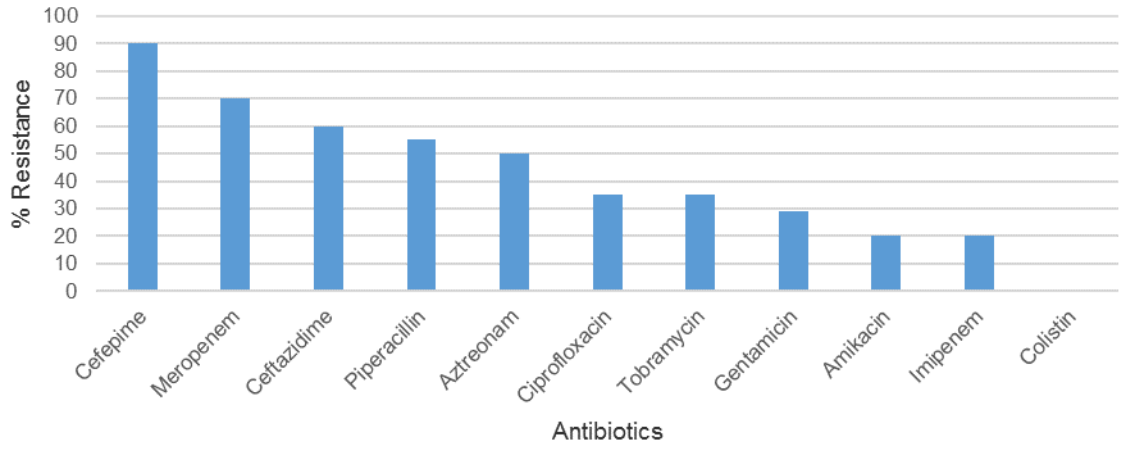

Figure 2: Percentage of bacterial resistance to different antibiotics used against *Pseudomonas aeruginosa*.

Table 2: Plasmid characterization isolated from *Pseudomonas aeruginosa*, showing numbers, sizes and patterns of resistance related to the antibiotics tested.

Isolates	Clinical source	Number of plasmids isolated	Size of plasmid (kb)	Resistant antibiotics
1	Urine	3	1.14, 1.4 and 25	FEP, MEM, CAZ, PIP, ATM, TOB
2	Pus	2	1.4 and 7.8	FEP, MEM, CAZ, PIP, TOB
3	Wound	2	1.4 and 22.8	FEP, MEM, CAZ, ATM
4	Burn	3	1.4, 7.4 and 23	FEP, MEM, PIP
5	Sputum	1	1.4	FEP. PIP, ATM
6	ATCC 25922	6	19.5, 16.0, 5.0, 4.9, 4.2, 2.0	-

increases the difficulty in eradicating this microorganism and leads to the presence of more persistent cases of infections. In addition, the production of enzymes by *P. aeruginosa* can lead to the inactivation of the antibiotics used for treatment (Thacharodi and Lamont, 2022). A study by Wright (2005) showed that *P. aeruginosa* possesses an inducible *ampC* gene encoding the hydrolytic enzyme β-lactamase; this enzyme can break the amide bond of β-lactam ring, leading to the inactivation of β-lactam antibiotics.

Other factors of resistance were acquired through the horizontal transfer of resistance genes from one bacteria to another or mutations in the bacterial chromosome (Breidenstein *et al*., 2011; Munita and Arias, 2016).

We extracted plasmids from the clinical isolates of *P. aeruginosa* followed by electrophoresis on an agarose gel to determine the number and size of the plasmids. The analysis of the gel showed that the isolates of *P. aeruginosa* harbored multiple copies of plasmids ranging from 0 to 3 with an estimated size range between 1 to 25 kb (Table 2). Our results are in correlation with other studies showing a similar pattern of antibiotic resistance of different strains of *P. aeruginosa* collected from patients with infected burns treated with fluoroquinolone-based antibiotics (Albaayit *et al*., 2018). Other researchers from India found that their *P. aeruginosa* isolates from similar specimens as ours showed a similar pattern of resistance to antibiotics (cefepime 65.96%, aztreonam 59.57% and piperacillin 61.7%) (Shaikh *et al*., 2015). Another study in 2013 from Egypt showed similar results to this study, where the percentage of resistance to cefepime was 98.2%, azteronam 82.5%, impineum 31.6% and amikacin 15.8% (Mahmmoud *et al*., 2013). A recent review conducted in 2021 on the use of antibiotics against *P. aeruginosa* suggested that using combinations of both cefepime and several other antibiotics, cefepime/taniborbactam, cefepime/enmetazobactam or cefepime/tazobactam as potential carbapenem-sparing agents with activity against ESBLs (Isler *et al*., 2021).

Many researchers have addressed the relationship between plasmid profiles and antibiotic resistance patterns in both Gram-positive and Gram-negative bacteria in an attempt to find correlations between the number of plasmids found and the type of antibiotic resistance. In our previous study on *S. aureus*, we did not find any correlation between the number of plasmids found and the pattern of resistance (Jaran, 2017); other studies on Gram-negative *E. coli* and *Salmonella* spp. also showed no direct correlation between the number of plasmids found and resistance patterns (Jaran, 2015; 2016). Several research studies showed that the presence of plasmids in *Pseudomonas* influenced the pattern of antibiotic resistance; they used plasmid curing followed by transformation into *E. coli* and demonstrated that resistance was transformed into it with similar molecular weight plasmids isolated from the transformed bacteria (Fatima *et al*., 2012; Jafari *et al*., 2013; Albaayit *et al*., 2018).

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CONCLUSION

The results data analysis in this study showed some correlation between the patterns of antibiotic resistance and plasmid profiles. Further studies should be conducted to determine the specific genes responsible for the resistance of *P. aeruginisa* from our isolates and from the plasmids they carry.

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CONFLICTS OF INTEREST

The authors have declared that no competing interests exist.

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