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Investigation of Virulence Factors in Resistance and Sensitive *Pseudomonas aeruginosa* Clinical Isolates

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Abstract

The pathogen "*Pseudomonas aeruginosa*" is extremely hazardous for people with weak immune systems because it has several virulence factors and can resist antibiotics. During the time period between October 2022 and February 2023, 102 specimens from different clinical sources, including burn, mid-stream urine, wound, ear, and sputum were collected. From 102 clinical specimens, only 33 isolates (32.35%) of *P. aeruginosa* were identified by Phenotypic characteristics, a biochemical test, the Vitek-2 compact system, and confirmed by Conventional Polymerase Chain Reaction (PCR). The antibiotic susceptibility test AST was done by the Vitek 2-Compact system. The highest resistance percentages were for Cephalosporin category at 81.8% (Cefotaxime, Ceftazidime, and Cefepime), while the lowest percentage was for the Fluoroquinolone category at 42.4% (Ciprofloxacin and Norfloxacin); 18 (54.5%) of the isolates were categorized as MDR. The production rates of virulence factors investigated in all tested isolates were 100% for protease, hemolysin, and swarming, while the lipase production rate was 48.5%.

Keywords: Protease, hemolysin, swarming, lipase and antibiotic patterns.

1. Introduction

Pseudomonas aeruginosa is an opportunistic gram-negative bacterium member of the ESKAPEE group of antibiotic-resistant pathogens (1). Hospital patients regard it as one of the primary causes of morbidity and mortality (2). It is responsible for a wide variety of serious illnesses, including cystic fibrosis and wound infections, endocarditis, urinary tract infections, ear infections, burns, nosocomial infections, bacteremia, pneumonia, and infections in people with impaired immune systems (3). A bacterial pathogen's typical infection cycle consists of the following stages: selecting and invading an appropriate host; dispersion throughout it; the appearance of illness signs; and, lastly, transmission to another host. The pathogen produces

several factors known as virulence factors to successfully complete each stage. Therefore, *P. aeruginosa* has multiple virulence factors link to both chronic and acute infections (4). The protease enzyme that catalyzes the breaking of a peptide or isopeptide bond (5) plays several roles in the pathogen's virulence, such as breaking down host physical barriers and facilitating colonization (4). It also has regulatory functions to enable the bacterium to respond to environmental changes and initiate infection at the right moment (6). Protease is also involved in biofilm formation, antibiotic resistance, and swarming motility (7). *P. aeruginosa* produces at least eight types of proteases, and these enzymes contribute to its pathogenicity (8).Lipase is a water-soluble enzyme that facilitates the breakdown of long-chain triglycerides into glycerol and fatty acids (9). *P. aeruginosa* produces two types of lipases, namely Lip A and Lip C. The lipases produced by bacteria may contribute to bacterial colonization and the development of diseases (10). Due to the degradation of dipalmitoyl phosphotidyl choline, a major lung surfactant lipid, and stimulating 12-hydroxyeicosatetraenoic acid (12-HETE) release from human platelets (11). Moreover, lipase can significantly inhibit monocyte chemotaxis, which is a function of cells that participate in the immune response (12). Hemolysin is an exotoxin that encourages the lysis of red blood cells, leading to the liberation of hemoglobin (13). Two hemolysins are produced by *P. aeruginosa*: rhamnolipid (RLs) heat-stable hemolysin and phospholipase C (PLC) heat-labile hemolysin (14). RLs are also essential for *P. aeruginosa* virulence, destroying immune cells and erythrocytes, twitching and swarming motility, protection, and biofilm formation. Additionally, they've been linked to the deterioration of individuals with pneumonia associated with ventilators as well as the breakdown and permeabilization of epithelial cells, which is essential for *P. aeruginosa* invasion (15). PLC is a virulence factor, especially for strains that colonize the human lung. It inhibits phagocytosis by blocking the neutrophil respiratory burst as it acts on substrates such as phosphatidylcholine and sphingomyelin. It also promotes bacterial invasion by producing cell membrane degradation, tissue deterioration, hemolysis (which aids iron release), and apoptosis (16). Swarming is a motion in which bacteria migrate collectively across a surface, powered by flagella and assisted by phenotypic adaptations (17). *P. aeruginosa* uses this motion as an essential virulence factor to invade novel sites (18) and develop biofilm (19). It is associated with increased virulence and antibiotic resistance to different antibiotics (20). This study investigated the relationship between MDR and sensitive isolates producing specific virulence factors. Understanding bacterial virulence behavior and relationships could pave the way for novel targets for MDR infection treatment.

2. Materials and Methods

2.1. Isolation and Identification of *Pseudomonas aeruginosa*:

102 different specimens were collected using sterile cotton swabs from wounds, burns, mid-stream urine, ear infections, and sputum specimens between October 2022 and February 2023. We used standard microbiological assays, including growth on MacConkey agar, growth on cetrimide agar, catalase and oxidase tests, Gram stain, and growth tests at 42 °C, to identify the bacterial isolates as *P. aeruginosa*. We tested the Vitek 2-Compact, a superior colorimetric technique for bacterial identification, using a Gram-negative (GN) card that includes 48 biochemical assays used in germ

diagnostics. We employed conventional PCR (16S rRNA) to confirm the identity of the bacterial isolate.

2.1.1. Extraction of DNA

The EasyPure® Genomic DNA Kit (Transgene®, China) was used to extract DNA from 33 *P.aeruginosa* isolates. The high selectivity test Qubit 4.0 (ThermoFisher®, USA) was used for estimating the concentration of DNA extracted from the isolates and the suitability of the specimens for use in downstream processes. Furthermore, the isolated DNA was stored at -20°C before use.

2.1.2. PCR amplification

Conventional PCR performed the amplification of the 16S rRNA gene. A PCR mixture with a total volume of 24 microliters was prepared and consisted of 1µl of forward primer) 5`-ACTCCTACGGGAGGCAGCAGT-3` ((10pmol /µl) (Macrogen, Korea), 1µl of reverse primer)5`- TATTACCGCGGCTGCTGGG-3`) (10pmol /µl) (Macrogen, Korea), 12.5 µl Master Mix (NEB®, England), 3 µl of template DNA, and 7.5 µl of Nuclease-free water (NEB®, England). The mixture was thoroughly mixed and vortexed to ensure homogeneous contents. The cycling program was adjusted as follows: an initial denaturation at 94°C for 5 minutes, followed by 30 cycles at 94°C for 30 seconds, 45 seconds at 50°C, 45 seconds at 72°C, and 7 minutes for the final extension at 72°C.

2.2. Test for antibiotic susceptibility

The antibiotic susceptibility test for *P. aeruginosa* was tested utilizing the Vitek 2-Compact system and the AST-N204 card. For 33 clinical isolates, susceptibility, resistance, and MIC measurements and interpretations were automatically recorded.

2.3. Detection of virulence factors

2.3.1. Protease production

To determine the proteolysis of bacterial isolates, a bacterial suspension was prepared by incubating a single colony of each isolate in five ml of nutrient broth for 18 hours at 37°C. Ten to twenty microliters of each suspension were transferred to skimmed milk agar (HiMedia/ India). The plates were incubated for 24–48 hours at 37°C. The presence of a clear zone indicates protease production (21).

2.3.2. Lipase production

Tween 20 agar, prepared according to Al Mohaini *et al*.'s procedure (22), was used to examine the ability of the bacterial isolates to produce the lipase enzyme. The plates were then incubated for 24 hours at 37 °C. The presence of a halo-clear zone surrounding bacterial growth shows that the enzyme was produced.

2.3.3. Hemolysin production

To detect the isolate's ability to produce hemolysin, the plate method was used. Each isolate was streaked on to 5% human blood agar (Mast Group/UK), and the Petri dishes were incubated at 37 °C for 24 hours. The presence of a clear, transparent halo around the growing colonies indicates their ability to produce the enzyme (23).

2.3.4. Swarming

The bacterial isolates swarming motility test was done on 5% human blood agar (Mast Group, UK). Then the plates were incubated for 24–48 hours at 37°C. The spread from the initial point of inoculation indicates swarming ability (24).

2.4. Statistical analysis:

For data entry and analysis, the Statistical Package for the Social Sciences (SPSS) version 21, Software, San Diego, California, USA, application was used. To establish the significance of the categorical variables, the Chi-square test was performed. $P<0.05$ was regarded as statistically significant, whereas $P<0.01$ was regarded as extremely significant.

3. Results and Discussion

Thirty-three specimens out of 102 bacterial specimens collected from patients referred to three hospitals in Baghdad were diagnosed as *P. aeruginosa*, as shown in **Figure 1**, agreeing with Rashad *et al*.'s result (25), who reported that out of 220 clinical specimens that were collected, only 33 isolates were recognized as *P. aeruginosa*.

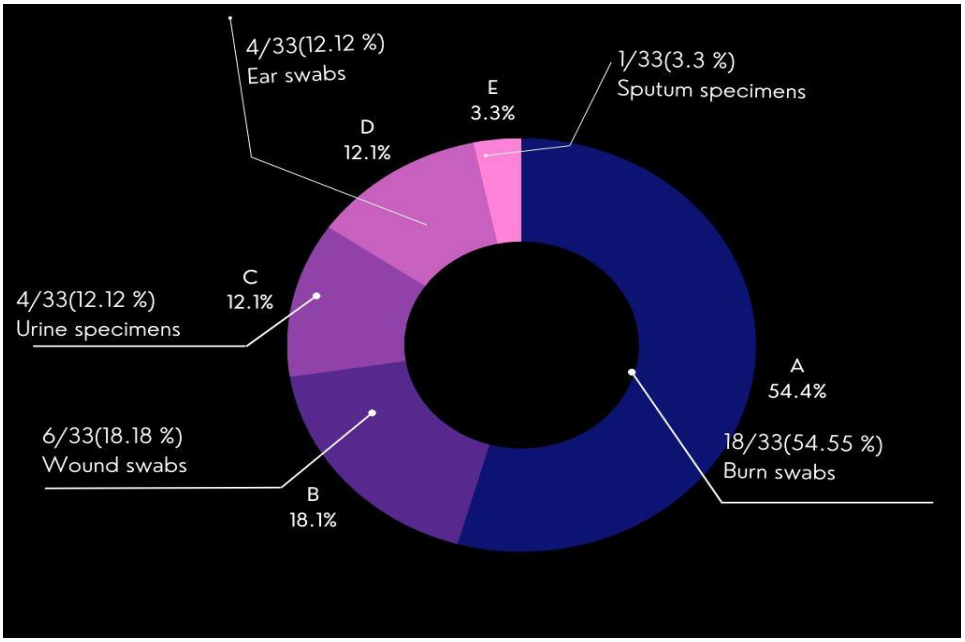
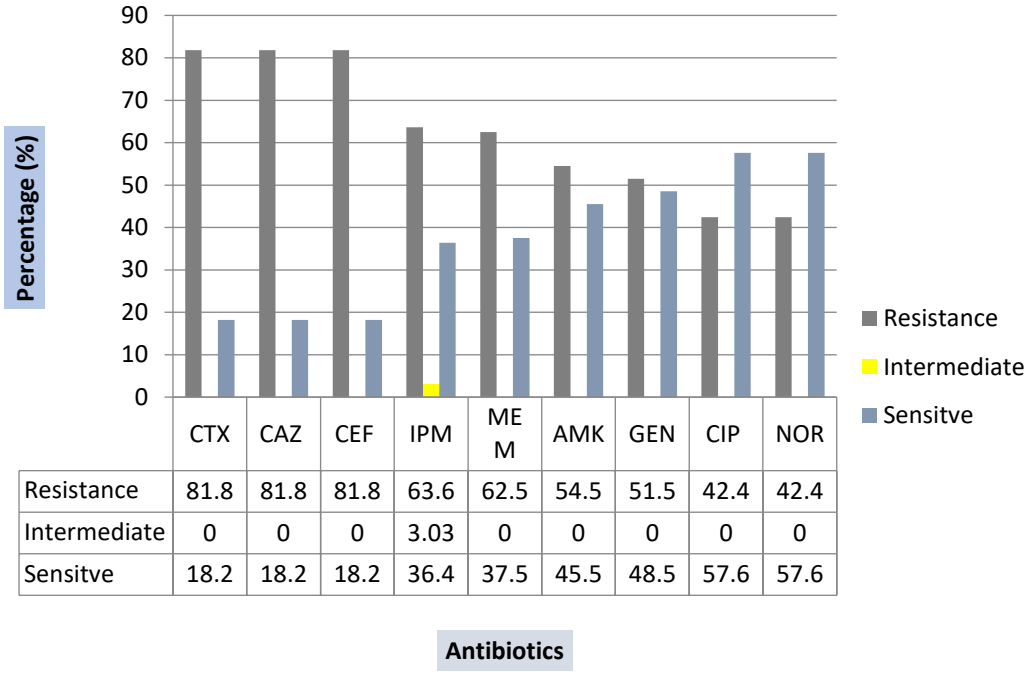


Figure 1. Isolation percentage of bacteria according to specimen source.

This study revealed *P. aeruginosa*'s highest prevalence in burn infections compared with other clinical specimen sources. This outcome coincides with previous studies (25). Because burns cause skin degeneration, which is the anatomical barrier and the body's first line of defense (26), burn injuries are more susceptible to opportunistic bacterial colonization by both endogenous and exogenous bacteria (27).

P. aeruginosa formed circular, mucoid, and smooth pale colonies with a sweaty grape odor on MacConkey agar, and the bacterial colonies appeared greenish-yellow on Cetrimide agar. We observed positive results from catalase and oxidase tests. Gram-negative bacilli were revealed by Gram staining, and the Vitek 2-test demonstrated that 34 specimens had up to a 95% probability

of *P. aeruginosa*. Previous studies have demonstrated that the VITEK 2 diagnostic approach is faster and less labor-intensive than traditional biochemical met Conventional and molecular identification methods, utilizing the 16S rRNA gene, proved 33 previously identified isolates to be *P. aeruginosa.* Polymerase chain reaction (PCR) amplified the 16S rRNA gene, enabling more accurate and sensitive bacterial identification (29).



	CTX	CAZ	CEF	IPM	MEM	AMK	GEN	CIP	NOR
Resistance	81.8	81.8	81.8	63.6	62.5	54.5	51.5	42.4	42.4
Intermediate	0	0	0	3.03	0	0	0	0	0
Sensitve	18.2	18.2	18.2	36.4	37.5	45.5	48.5	57.6	57.6

Figure 2. Antibiotic resistance frequency of *P. aeruginosa* isolates, Cefotaxime (CTX), Ceftazidime (CAZ), Cefepime (CEF), Imipenem (IPM), Meropenem (MEM), Amikacin (AMK), Gentamicin (GEN), Ciprofloxacin (CIP), and Norfloxacin (NOR).

The antibiotic susceptibility of all 33 *P. aeruginosa* isolates to nine antibiotics from four different antimicrobial categories was assessed using the VITEK 2 compact system. The data were subsequently interpreted using the Clinical and Laboratory Standards Institute 2022 (CLSI). In the current study, the highest frequency of resistance was shown to be against Cephalosporin antibiotics, while the least resistance was against Fluoroquinolone antibiotics. As shown in **Figure 2** The percentage of resistant isolates to cephalosporin antibiotics (Cefotaxime, Ceftazidime, and Cefepime) was 81.8%. This result aligns with the ceftazidime resistance rate of 81% reported in previous studies (30), as well as the Cefotaxime resistance findings (31). While Cefepime resistance was close to the outcome of the Iraqi study by (32), which was 77.2%, the elevated resistance of *P. aeruginosa* isolates to cephalosporin antibiotics is expected, as revealed by previous results like (31), who found an elevated resistance rate to cephalosporin in *P. aeruginosa* isolates. Cephalosporins are antibiotics that belong to the beta-lactam class (33). They hinder the production of the bacterial cell wall, particularly the peptidoglycan bacterial exoskeleton. Bacterial

resistance to beta-lactams by β-lactamase enzymes, which break down the β-lactam ring, renders antibiotics inactive (34).

The current results showed that the percentage of resistant *P. aeruginosa* isolates to the Carbapenem antibiotics Imipenem and Meropenem was 63.6% and 62.5%, respectively. However, the difference in result is apparent compared with preceding study findings as (35), who found *P. aeruginosa* resistance rates of 11.11% and 23.45% for Imipenem and Meropenem, respectively. The Carbapenem antibiotic category belongs to the beta-lactam class. The primary target of Carbapenem is During bacterial cell wall production, "transpeptidase inhibition enzyme" activity prevents peptide cross-linkage action, resulting in a rise in autolytic activity and, eventually, cell death. The resistance to this category is intrinsic to certain species and is comprised of the ability to resist antibiotic action through a variety of mechanisms, including the absence of a specific target, a fundamental difference in the structure of the cytoplasmatic membrane, a refusal to penetrate the outer membrane, or carbapenemase production (36).

In this research, *P. aeruginosa's* sensitivity to Amikacin and Gentamicin belonging to the aminoglycoside group has shown notably moderate resistance (54.5% and 51.5%, respectively). These findings disagreed with a local study by (37), who discovered that *P. aeruginosa* isolates have a low resistance to Amikacin and Gentamicin (25% and 31.3%, respectively). The aminoglycosides block the manufacture of proteins in bacterial cells by linking with the 30S component of the ribosome, and *Pseudomonas* sp. became resistant to aminoglycosides mostly as a result of changes in target enzymes and antibiotic deactivation (38).

Concerning Fluoroquinolone antibiotics, the results revealed *P. aeruginosa* isolates had 42.4% resistance rates for Ciprofloxacin and Norfloxacin, which are higher than the resistance rates of Ciprofloxacin (20%) and Norfloxacin (16.7%), according to results conducted by (39). Fluoroquinolones inhibit bacterial topoisomerase enzymes, which are responsible for DNA strand breakage and reconnecting, thereby decreasing pressure in supercoiled DNA during DNA replication (40). *P. aeruginosa* become resistant to fluoroquinolones in a number of ways, including changes in the genes that code for the bacterial DNA topoisomerase. This is the most common way that *P. aeruginosa* isolates became resistant to fluoroquinolones. Overexpression of active efflux systems, on the other hand, can lower membrane permeability. Also, plasmids that have the extended-spectrum beta-lactamase (ESBL) gene can also have the quinolone resistance gene on them. This may explain why ESBL-producing bacteria have a high level of quinolone resistance (41).

The researchers identified 18 (54.5%) of the 33 *P. aeruginosa* isolates as multidrug-resistant *P. aeruginosa*, which means the isolate was resistant to at least one antibiotic across three antimicrobial categories (42), while the remaining 15 isolates (45.4%) were non-MDR. These results agree with local research by (43), which revealed that 60% of *P. aeruginosa* was MDR.

We investigated the production of virulence factors for all *P. aeruginosa* isolates, and the results showed that all isolates produce hemolysin. This research aligns with (44), which demonstrated that all *P. aeruginosa* isolates had a 100% capacity to produce hemolysin. Protease detection demonstrated that all 33 *P. aeruginosa* isolates were able to produce proteases. This result surpasses the 86% protease activity rates reported in the Egyptian study by (45). Swarming investigations for *P. aeruginosa* isolates revealed that all isolates are able to swarm on blood agar,

the same result detected by (46). The lipase enzyme detection results show that out of the 33 *P. aeruginosa* isolates tested, 16 (48.5%) produced the lipase enzyme. This finding disagrees with (47), which showed that only 16% of bacterial strains were capable of producing lipase. The presence of these virulence factors assists in bacterial attachment, colonization, and immunosuppression in order to weaken and infect the host. A combination of these virulence characteristics can be lethal and effective in defeating the host defense system (48). The relation between virulence factors present in sensitive and resistant *P. aeruginosa* isolates is given in **Table 1.**

Table 1. The distribution of virulence factors of sensitive and multidrug resistance *P. aeruginosa* isolates

Virulence factors		**Antibiotic resistance**				
		Sensitive		**MDR**		***p-value***
		N	**%**	**N**	**%**	
Hemolysin	**Low**	2	13.3%	0	0.0%	-
	Medium	8	53.3%	7	38.9%	0.144
	Strong	5	33.3%	11	61.1%	0.004**
	Total	15	45.45%	18	54.55%	0.317
Protease	**Low**	3	20.0%	2	11.1%	0.106
	Medium	5	33.3%	5	27.8%	0.522
	Strong	7	46.7%	11	61.1%	0.178
	Total	15	45.45%	18	54.55%	0.317
Lipase	**Non- produce**	7	46.7%	10	55.6%	0.375
	Low	3	20.0%	3	16.7%	0.622
	Medium	3	20.0%	2	11.1%	0.106
	Strong	2	13.3%	3	16.7%	0.465
	Total	15	45.45%	18	54.55%	0.317
Swarming	**Low**	2	13.3%	0	0.0%	-
	Medium	9	60.0%	4	22.2%	<0.001**
	Strong	4	26.7%	14	77.8%	<0.001**
	Total	15	45.45%	18	54.55%	0.317

(Non- produce, Low, Medium and Strong), levels of activity based on diameter of zone around well; Total, total number of isolates expressing specific virulence factor.

**: highly significant difference

The results demonstrate that MDR isolates produce virulence factors more frequently but insignificantly differently than sensitive isolates. MDR isolates significantly differ from sensitive isolates in both the strong intensity of hemolysin production and the medium and strong swarming levels. The results of the present study agree with previous studies, which mention no significant difference in results between *P. aeruginosa* MDR and non-MDR isolates in terms of various virulence factors such as hemolysin, proteases (46), lipase (45), and swarming (49). Meanwhile, other studies reported the presence of these virulence factors associated with increases in antibiotic resistance (20, 50).

4. Conclusion

To summarize, the present research provides insight into the incidence of antibiotic resistance in *P. aeruginosa* clinical isolates and demonstrates the non-differences in producing some virulence factors between MDR and sensitive *P. aeruginosa* isolates.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Ethical Clearance

The study was conducted after receiving agreement from the participants and ethical approval from the biology department's ethics committee at the University of Baghdad's College of Science (CSEC/0922/0072) on September 25, 2022. and this is consistent with the instructions of the Iraqi Ministry of Health and Environment.

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