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Incidence of Virulence Genes (*Phzm, phzs*) and Antibiotic Resistance Presence in *Pseudomonas aeruginosa* Strains Isolated from Hospitalized Patients in Baghdad City

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Abstract: *Pseudomonas aeruginosa*, a prominent culprit of hospital-acquired infections, is progressively linked to epidemics and poses a global challenge across diverse medical facilities. The aim of the study to emergence of multidrug-resistant (MDR) *P. aeruginosa* has assumed paramount clinical importance, from October 2022 to January 2023, 150 specimens were collected from patients referring to the medical City hospitals in Baghdad and a private laboratory in Baghdad. The strains were isolated from clinical specimens: 46 burn swabs, 43 urine sample, 8 Blood specimens, 39 sputum sample, 12 wound swabs and 2 ear swabs of patients, these samples were taken from patients of different ages, men, women and children, all samples underwent bacteriological and biochemical examinations for diagnosis. Out of the total 150 specimens, 38 (25.33%) were identified as *Pseudomonas aeruginosa*, while the remaining 112 (74.67%) exhibited different bacterial strains. The *P. aeruginosa* isolates were verified using the VITEK2 system and demonstrated positive outcomes for oxidase, catalase, and hemolysin production. The pyocyanin test results were mixed, ranging from negative to positive, and confirm diagnosis tests the ability to grow at 42°C conducted for further confirmation (heat tolerance). It was concluded That PCR analysis was performed on 38 isolates, targeting the *16SrRNA* (housekeeping gene) as well as the virulence genes *Phzm, Phzs*. The findings revealed that all samples tested positive for the *16SrRNA* gene. And also, 36 (94.74%) were positive for *Phzm*, 31 (81.58%) for *Phzs*.

Keywords: *Pseudomonas aeruginosa*, *Phzm, phzs*.

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Introduction

Pseudomonas aeruginosa, a Gram-negative aerobic bacterium, can be found in diverse environments, ranging from soil and plants to mammal tissues(1). Its ability to persist on medical devices and various surfaces is facilitated by essential binding elements like flagella, pili, and biofilms (2). This microorganism has gained prominence as a significant cause of hospital-acquired infections and is associated with the escalating issue of antibiotic resistance(3).

Pseudomonas aeruginosa is notably linked to healthcare-related infections, encompassing conditions such as ventilator-associated pneumonia (VAP), intensive care unit infections, bloodstream infections from central lines, surgical site infections, urinary tract infections, burn infections,

keratitis, and otitis media(4). These infections frequently result in high mortality rates among afflicted patients(5). The adaptability of *P. aeruginosa* to changing environmental conditions contributes to its swift development of antibiotic resistance(6).

The selection of appropriate antibiotics for patients with *Pseudomonas aeruginosa* infections is notably challenging due to the inherent resistance of this pathogen to many commercially available antibiotics. The prevalence of multidrug-resistant strains further complicates treatment(7), often necessitating the use of novel or "last resort" agents like Colistin.

Pseudomonas aeruginosa is a versatile and dangerous pathogen known for causing various infections, especially in people with weakened immune systems. It deploys several virulence factors, including exotoxins, proteases, siderophores, and flagella, to enhance its disease-causing capabilities(8).

Exotoxins like pyocyanin and exoenzyme S damage host tissues and disrupt cellular signaling (9). Proteases, such as elastase, alkaline protease, and LasA protease, break down host proteins and contribute to tissue damage(10). Siderophores like pyoverdine and pyochelin help the bacterium acquire iron for survival in the host (11). Flagella enable *P. aeruginosa* to move toward and attach to host cells, aiding colonization and infection (12).

The genes known as *phzM* and *phzS* play a crucial role in the synthesis of the phenazine virulence factor in *Pseudomonas aeruginosa*(13).

Additionally, these genes have been associated with the formation of biofilms, another important virulence factor of this bacterium. They are also responsible for producing pyocyanin, a virulence factor that contributes to the severity of P. aeruginosa infections(14).

Research has demonstrated that the absence of *phzM* and *phzS* genes results in reduced pyocyanin production and decreased virulence of the bacterium(15).

Consequently, targeting these genes could be a promising avenue for developing new therapies to treat *P. aeruginosa* infections (16).

Materials and methods

Specimens collection

Between October 2022 and January 2023, a total of 150 specimens were gathered from patients who sought medical care at both the Medical City hospitals and a private laboratory in Baghdad. These specimens were drawn from various clinical sources: 46 from burn swabs, 43 from urine samples, 8 from blood specimens, 39 from sputum samples, 12 from wound swabs, and 2 from ear swabs. The patient pool covered diverse demographics, encompassing individuals of different ages, genders, including men, women, and children.

Bacterial identification

Bacterial identification was initiated by assessing the morphology of colonies on Nutrient agar, MacConkey agar, blood agar and Cetrimide agar (17). This involved observing the shape, texture, and color of the colonies (Figures 1, 2).

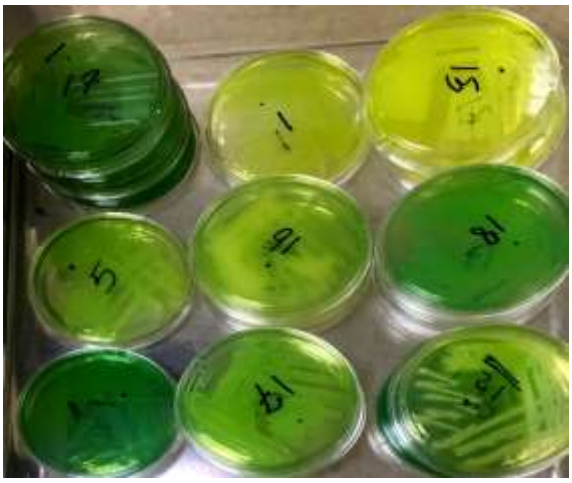

Figure (1): *Pseudomonas aeruginosa* colonies on Nutrient agar after 24 hours incubated.

Figure (2): *Pseudomonas aeruginosa* colonies on Cetrmide agar after 24 hours incubated.

The determination of minimum inhibitory concentration (MIC) values for antibiotics was executed through the utilization of the VITEK 2 Compact System

This advanced system enabled the assessment of MIC values for a range of thirteen distinct bioactive agents, encompassing Amikacin, Cefepime, Ceftazidime, Ciprofloxacin, Piperacillin, Meropenem, Imipenem, Gentamicin, Colistin, Ticarcillin/Clavulanic Acid, Ticarcillin, Piperacillin/Tazobactam, and Tobramycin. This process was accomplished by employing specialized cards tailored for Antimicrobial Susceptibility Testing (AST) examination. The implementation adhered to the comprehensive guidelines set forth by Pincus (2006) as well as the meticulous instructions provided by BioMérieux, the manufacturer of the system the measurements obtained were subsequently subjected to interpretation in accordance with the directives laid out by the Clinical and Laboratory Standards Institute (CLSI 2022) As in the (Table 1)**.**

Table (1): Antibiotics Disks using in this study.

Antimicrobial	MIC	Interpretation
Ticarcillin	≤16	TI
Ticarcillin/Clavulanic acid	≤16/2	TIM
Piperacillin/Tazobactam	≤16/4	TZB
Piperacillin	≤16	PRL
Ceftazidime	≤8	CAZ
Cefepime	≤8	FEP
Imipenem	≤2	IPM
Meropenem	≤2	MEM
Amikacin	≤16	AK
Gentamicin	≤4	CN
Tobramycin	≤4	TOB
Ciprofloxacin	≤1	CIP
Colistin	≤2	CT

Genetic identification

DNA extraction from pure cultures of *Pseudomonas aeruginosa* bacteria was carried out using the EasyPure® Bacteria Genomic DNA Kit protocol. The resulting PCR products were visualized through electrophoresis on a 1.2% agarose gel stained with Red Safe.

The primer pairs used in this study were prepared in accordance with the manufacturer's instructions. They were dissolved in sterile double-distilled water (ddH2O). A stock solution with a concentration of 100 pmol/µl was prepared either by adding nuclease-free water or reconstituting lyophilized primer vials with ddH2O. Subsequently, a working stock solution with a concentration of 10 pmol/µl was created by mixing 10 µl of the stock primer solution with 90 µl of ddH2O. Details of the specific primers utilized in this study are provided in (Table 2).

In this research, 38 isolates underwent PCR analysis for the detection of the *16SrRNA* gene (a housekeeping gene) as well as the virulence genes *phzm, phzs* (Figures 3,4). PCR reactions were conducted in a final volume of 25 µl. Each reaction consisted of 12.5 µl of master mix, 1.5 µl of forward primer, 1.5 µl of reverse primer, 4.5 µl of nuclease-free water, and 5 µl of template DNA and in conditions of special thermal cycling reactions for gene detection (Table 3). The amplified PCR products were visualized using agarose gel electrophoresis, and a DNA marker (Neogen/USA) was included with each gel run.

Table (2): Sequencing and PCR Conditions in this study.

No.	Secretion System	Primer	Sequence 5 to 3	Product	Reference
1	***Psedo-16s***	F	5-CCTACGGGAGGCAGCAG-3	215bp	(18)
		R	5-ATTACCGCGGCTGCTGG-3		
2	***PHzM***	F	5-CAAGTTGTTACCGGGGAATG-3	172bp	(19)
		R	5-AGATCTCGAAGGCCACCAG-3		
3	***PHzS***	F	5-GGAAAGCAGCAGCGAGATAC-3	206bp	(19)
		R	5-AGTACTGCGGATAGGCGTTG-3		

Table (3): Thermo cycling reactions for detection genes in the study.

Gene name	Temperature (°C)/Time					Cycle number
	First denaturation	Condition of cycling			Last extension	
		Denaturation	Annealing	Extension		
16srRna	94/5 min	95/0.5 Min	54/ 0.5 Min	72/1 min	72/10 Min	35
PHzm	94/5 min	95/0.5 Min	54/0.5 Sec	72/1 min	72/10 Min	35
PHzs	94/5 min	95/0.5min	54/ 0.5 Min	72/1 min	72/10 Min	35

Results and discussion

Between October 2022 to March 2023, a comprehensive set of 150 clinical specimens was meticulously gathered from a diverse group of patients, encompassing individuals of all ages and genders. These specimens hailed from various sources, including wound, burn, sputum, and urine samples, each representing patients exhibiting distinct clinical symptoms. To ensure the preservation of sample viability and integrity during their transit, they were promptly placed in appropriate transport media. The application of transport media serves the crucial purpose of safeguarding the samples and conserving their original microbial composition until their arrival at the laboratory for subsequent processing.

Upon reaching the laboratory, these collected specimens underwent rigorous isolation and identification procedures. In this study, a total of 150 samples that displayed positive growth were isolated from clinical specimens derived from a wide array of infectious sources, including wounds, burns, urine, and sputum. These samples were subjected to conventional microbiological detection techniques, involving the utilization of various enrichment, differential, and selective media. The bacterial isolates underwent rigorous identification through a battery of bacteriological and biochemical tests. Among the 150 isolates scrutinized, a definitive total of 38 isolates were unequivocally identified as *Pseudomonas aeruginosa*. The remaining isolates were found to be distributed among gram-positive and gram-negative bacteria, representing various other bacterial species (Table 4).

Table (4): Incidence *of Pseudomonas aeruginosa* Isolates Across Clinical Samples.

Source	No .of samples	No. of samples with bacterial growth	No. of *P. aeruginosa* isolates
Wounds	12	9 (75 %)	3 (25 %)
Burns	46	28 (60.9 %)	18 (39.1 %)
Sputum	39	31 (79.48 %)	8 (20.52 %)
Urine	43	35 (81.39 %)	8 (18.61 %)
Blood	8	7 (87.5 %)	1 (12.5 %)
Ear swab	2	2 (100 %)	0 (0 %)
Total	150	112 (74.7)	38 (25.33)

Note: that the percentages in parentheses indicate the proportion of positive samples within each category.

Evaluate antibiotic susceptibility of *Pseudomonas aeruginosa* through VITEK2 compact system for minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of antibiotics for *38 Pseudomonas aeruginosa* isolates was determined using the VITEK 2 Compact system (20). Samples were standardized, loaded into the device, and assessed overnight using a specific card designed for antimicrobial susceptibility testing (AST). This allowed testing of 13 different antibiotics (21).

In this study, the MIC values of thirteen antibiotics against *Pseudomonas aeruginosa* isolates were determined using the Vitek2 Compact system and specific cards designed for antimicrobial susceptibility testing (AST). The findings showed varying degrees of resistance, sensitivity, and in some cases, intermediate susceptibility for these antibiotics (Table 5).

Table (5): Antimicrobial Susceptibility of 38 Pseudomonas aeruginosa Isolates to Different Antimicrobial Agents

Antibiotic	Resistant	Intermediate	Sensitive
Ticarcillin	28 (73.68%)	0 (0.0%)	10 (26.32%)
Ticarcillin/Clavulanic acid	4 (10.53%)	0 (0.0%)	34 (89.47%)
Piperacillin/Tazobactam	11(28.95%)	0 (0.0%)	27 (71.05%)
Piperacillin	7 (18.42%)	0 (0.0%)	31 (81.58)
Ceftazidime	9 (23.68%)	0 (0.0%)	29 (76.32%)
Cefepime	5 (13.16%)	5 (13.16%)	28 (73.68%)
Imipenem	23 (60.53%)	0 (0.0%)	15 (39.47%)
Meropenem	7(18.42%)	2 (5.26%)	29 (76.32%)
Amikacin	7 (18.42%)	0 (0.0%)	31 (81.58%)
Gentamicin	5 (13.16%)	20 (52.36%)	13 (34.21%)
Tobramycin	11 (28.95%)	9 (23.68%)	18 (47.37%)
Ciprofloxacin	25 (65.79%)	5 (13.16%)	8 (21.05%)
Colistin	14 (36.84%)	0 (0.0%)	24 (63.16%)

These MIC values provide valuable insights into the effectiveness of these antibiotics against *Pseudomonas aeruginosa* isolates, with variations in resistance and sensitivity profiles among the antibiotics tested.

In this study, the highest rate of resistance was observed against Ticarcillin, with a prevalence of 73.64%. This was followed by ciprofloxacin, which exhibited a resistance rate of 65.76%, and imipenem, with a resistance rate of 60.53%. On the other hand, the lowest resistance rate was associated with Clavulanic Acid/Ticarcillin, with only 10.53% resistance, followed by gentamicin and cefepime, both having a resistance rate of 13.16%. The remaining antibiotics showed resistance rates below the average when tested against *Pseudomonas aeruginosa*.

When comparing the results of our study with those of another study conducted in 2016, it was observed that in the Kingdom of Saudi Arabia, 88% of Ticarcillin isolates of *P aeruginosa* exhibited resistance to the antibiotic. This suggests a relatively high level of resistance to Ticarcillin among *P aeruginosa* isolates in that region (22).

In another study conducted in Iraq by Ismail, *et al.* (23), their findings revealed the antibiotic susceptibility patterns of *P. aeruginosa* isolates. The results indicated high rates of sensitivity to the cephalosporin group, including cefepime (86.4%) and ceftazidime

(90.9%), as well as piperacillin-tazobactam (90.9%). Colistin (81.8%) and polymyxin (86.4%) also showed relatively high levels of sensitivity. Moreover, the isolates displayed sensitivity to amikacin (81.8%) and gentamicin (72.2%). Sensitivity rates to carbapenem antibiotics, specifically imipenem, meropenem, and doripenem, were 73.9%, 68.2%, and 81.8%, respectively.

Therefore, our conclusion is that there is a notable rise in antibiotic resistance among *P aeruginosa*, with a particularly concerning increase in resistance to colistin(23).

In an Our study did not match with (Hakemi Vala *et al*. 2014) in Tehran, Iran, *P. aeruginosa* isolates from burn patients showed the highest rate of resistance to aztreonam, ceftriaxone, and cefotaxim (82.98%) and to gentamycin, ceftazidime, and piperacillin (95.1%)(24).

Molecular detection of target genes

In this study, all of the samples, which amounted to 38 in total, exhibited a positive result for the presence of the *16S_rRNA* gene, representing a 100% detection rate. Additionally, the *Phzm* gene was detected in 36 samples, accounting for a 94.74 % detection rate Our study aligns with research conducted in Erbil City and close to the percentage of our study, where it was observed that 92.5% of *P. aeruginosa* isolates contained the *phzm* gene. Our findings indicate a consistent presence of this gene among isolates in Iraq, suggesting a degree of genetic uniformity among *P. aeruginosa* isolates within the region(25).

While the *phzs* gene was positively identified in 81.58 % of the total samples in a different study conducted in southern Iraq, the *phzs* gene was identified in *P. aeruginosa* at a rate of 77%, which is slightly lower than the percentage we observed for this gene in our study (26).

The accuracy of the PCR results was further confirmed by comparing the molecular weight of the amplified products to that of a 100bp DNA ladder. This comparison was facilitated by analyzing the bands on agarose gel electrophoresis, revealing a distinct single amplicon band in the PCR product. These results were visualized under UV violet light using Red Safe, a DNA-specific dye, with the template DNA manifesting as a solitary band (Figures 3, 4).

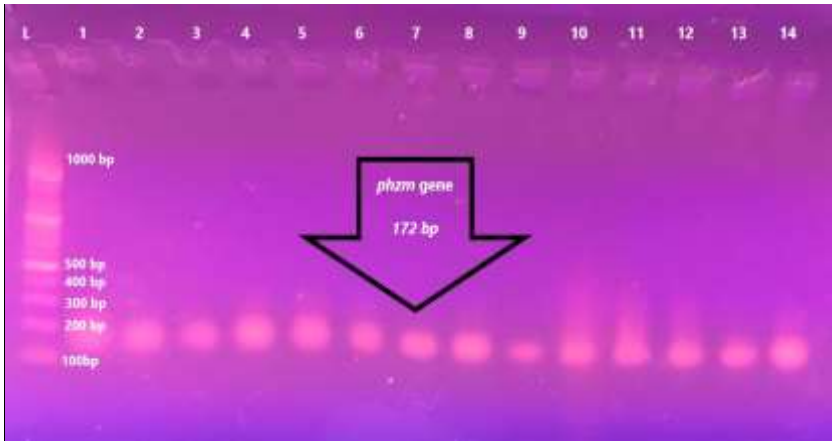

Figure (3): Gel electrophoresis of amplified PCR product of *phzm* gene in PCR at 70v for 90 min and 1.2% agarose stained with Red Safe. DNA ladder (100bp).

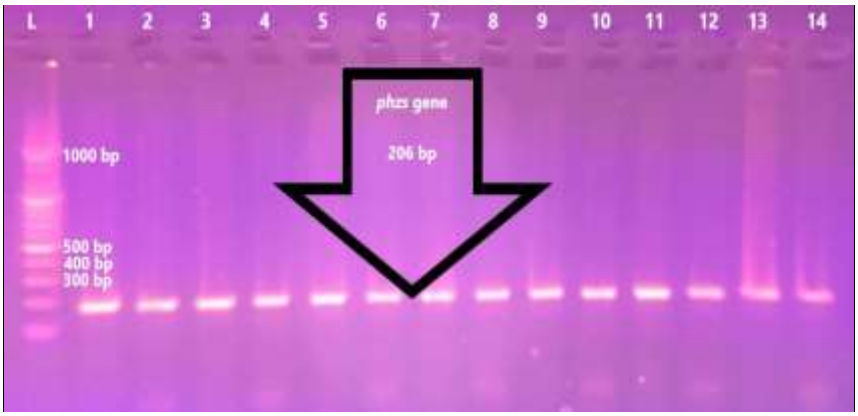

Figure (4): Gel electrophoresis of amplified PCR product of *phzs* gene in PCR at 70v for 90 min and 1.2% agarose stained with Red Safe. DNA ladder (100bp).

Conclusion

This study highlights the prevalence of virulence genes associated with pyocyanin production in *Pseudomonas aeruginosa* strains among patients from different medical facilities in Baghdad. Additionally, it underscores the resistance of this bacterium to various antibiotics. These findings underscore the critical need for a re-evaluation of antibiotic treatment strategies for patients in this region

Understanding these virulence factors is crucial for developing effective treatments and preventive measures against *P. aeruginosa* infections.

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