THE DETECTION OF *PHZS* AND *PHZM* GENES IN PYOCYANIN FORMING AND ANTIBIOTIC RESISTING *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES

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ABSTRACT

This study was aimed to detect the *phzS* and *phzM* genes in *Pseudomonas aeruginosa* clinical isolates that form pyocyanin and resist antibiotics. The study was conducted by collecting 240 samples from different clinical sources (burns, wounds, urinary tract, and ear infections) at Medicine City laboratories Baghdad-Iraq. Only 140 samples were diagnosed as *P. aeruginosa*, with the major identification depending on morphological characteristics, biochemical tests, the Vitek 2 compact system, and molecular detection of the *16S rRNA* gene responsible for this bacterium. Identified isolates were investigated for hemolysin, protease enzymes, and pyocyanin production. The genes *phzS* and *phzM*, involved in pyocyanin production, were investigated, and the isolates' sensitivity to 12 antibiotics was also tested. The results showed that all the isolates were able to produce hemolysin, 80% of the isolates were protease enzyme producers, and 72.15% produced pyocyanin. The presence percentage of the *phzS* gene (90%) was higher than that of the *phzM* gene (70%), and only the isolates that possessed the two genes producing pyocyanin, while those that contained one of the genes *phzS* or *phzM* did not. The highest levels of antibiotic resistance were for colistin (100%) and ceftazidime (97.14%), while the least were for imipenem (26.42%) and piperacillin-tazobactam (22.85%). The isolates producing pyocyanin are more resistant to antibiotics than those unable to produce it.

Keywords: pyocyanin genes, virulence factors, pathogenic bacteria, antibiotic resistance.

الكشف عن جينات *phzS* و *phzM* في العزلات السريرية للزوائف الزنجارية المكونة للبايوسيانين والمقاومة للمضادات الحيوية

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المستخلص

هدفت الدراسة الى الكشف عن جينات *phzM* و *phzS* في العزلات السريرية للزوائف الزنجارية المكونة للبايوسيانين والمقاومة للمضادات الحيوية. تضمنت الدراسة الحالية جمع 240 عينة من مصادرَ سريرية مختلفة (الحروق والجروح والمسالك البولية والتهابات الأذن) من مختبرات مدينة الطب بغداد–العراق. تم تشخيص 140 عينة على انها *P. aeruginosa* من خلال الاختبارات المورفولوجية و البيوكيميائية و نظام Vitek 2 والكشف الجزيئي عن جين *16S rRNA* المسؤول عن هذه البكتريا. اختبرت العزلات المشخصة عن قدرتها لانتاج الهيموليسين وإنزيمات البروتيز والبايوسيانين. و فحصت الجينات *phzS* و *phzM* المسؤولة عن إنتاج البايوسيانين واختبرت حساسية العزلات لـ 12 مضاد حيوي. اظهرت النتائج ان جميع العزلات كانت منتجة للهيمولايسين و 80٪ من العزلات كانت منتجة لانزيمات البروتييز و 72.15% كانت منتجة للبايوسيانين. ان نسبة وجود الجين *phzS* (90%) اعلى من الجين *phzM* (70%)، وان العزلات التي تمتلك كلا الجينين كانت منتجة للبايوسيانين، في حين أن العزلات التي احتوت على أحد الجينين لم تكن منتجة. أن أعلى مقاومة للمضادات الحيوية كانت للكوليستين (100٪) والسيفتازيديم (97.14٪)، بينما أقلها كانت للإمبينيم (26.42٪) وبيبراسيلين–تازوباكتام (22.85٪)، وكانت العزلات المنتجة للبايوسيانين أكثر مقاومة للمضادات الحيوية من العزلات الغير قادرة على الانتاج.

الكلمات المفتاحية: جينات البايوسيانين، عوامل الضراوة، بكتريا مرضية، مقاومة المضادات الحيوية.

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INTRODUCTION

P. aeruginosa is a common bacterial infection that affects humans, animals, and plants. From a mild, self-limiting illness to a catastrophic, incapacitating systemic disease with high morbidity and death, infections can vary in severity. *P. aeruginosa* is known to cause a wide range of illnesses in both fit people and people with compromised immune systems (37). This bacterium is one of the most significant kinds of bacteria that cause nosocomial infections, which are dangerous for hospital patients (7, 30). Where these bacteria displayed a propensity to grow on a variety of substrates. The bacterium has been discovered in several products, including soap, cleaning supplies, respiratory gear, beds, endoscopes, distilled water, and suction tools. Hospital environments are where it is most prevalent (31). It has broken down the host's defences and caused many diseases, represented by respiratory infections and infections of wounds, burns, skin, eye infections, urinary tract, infections of the middle ear, bacteraemia, infections of the bones and joints, and infections digestive system (22). This bacterium may grow at a variety of temperatures, growing at room temperature and 37˚C. Slower rates of growth were observed at 4˚C (4, 31). It can also grow at 42 °C, which sets it apart from other clinically relevant *Pseudomonas* species (4, 24, 36). The reason for its widespread distribution and capacity to cause severe injuries is related to its low nutritional requirements, resistance to a number of environmental conditions and antibiotics, as well as its various metabolic and enzymatic features. It is especially common in warm, humid areas (18). Thus, it's difficult to avoid encountering *P.* aeruginosa in the environment. Patients frequently contract opportunistic infections from hospital isolates, which typically exhibit higher antibiotic resistance than environmental samples (5, 35). Infections caused by *P. aeruginosa* bacteria are difficult to handle because of their susceptibility to certain antipseudomonal agents and their ability to develop antibiotic resistance determinants (39). *P. aeruginosa* produces several virulence factors such as biofilm, exopolysaccharides, elastases, pigments (pyocyanin and pyoverdine), proteases, lipases, and other toxins, including exotoxin A (12, 40). Many members of *P. aeruginosa* make pigments and possess the cytochrome oxidase enzyme, which is a phylogenetic taxonomic feature (6). It also produces other pigments, such as Pyorubrin (red), Pyomelanin (brown), and Pyoverdine (greens yellowish also called Pseudobactin) (25). Pyocyanin is a nitrogen-containing ($C_{13}H_{10}N_2O$) water-soluble extracellular phenazine derivative pigment formed as a secondary metabolite, and pyocyanin (from "pyocyanius") refers to "blue pus (29). Pyocyanin is also known for its bactericidal action against other bacteria, which reduces competition for survival and nutrients. As a virulence factor, it induces the cell to lyse in the host as it can quickly cross cell membranes due to its solubility. *P. aeruginosa* strains that do not produce pyocyanin have low pathogenicity and a higher susceptibility to the immune response (17). *P. aeruginosa* PAO1 consists of two homologous core loci (operon *phzA1B1C1D1E1F1G1* and *phzA2B-2C2D2E2F2G2*) to code for phenazine-1-carboxylic acid and two phenazine genes (*phzM* and *phzS*) responsible for converting the enzymes of phenazine-1-carboxylic acid to pyocyanin (20). All the pyocyanin-producing isolates carried the phenazine biosynthetic operon *phzA-G* gene and two phenazine-modifying genes, *phzS* and *phzM*. *phzM* encodes a protein (36.4 kDa) that most closely resembles the O-methyl transferases of bacteria. *PhzS* encodes a protein (43.6 kDa) similar to monooxygenases in bacteria. Among the pseudomonads, only *P. aeruginosa* has been found to contain two copies of the phenazine operon (26). The production of Pyocyanin and its role as a virulence factor have been the topic of numerous investigations, yet little is known about the genes that are responsible for pyocyanin production. Also, few have identified the genes *phzS* and *phzM* and their presence and association with productivity and the effect of antibiotic resistance, and this is the main objective of our current study.

MATERIALS AND METHODS

Sample collection: Clinical samples were collected between October 1, 2022, and

December 12, 2022, from Medicine City laboratories. The 240 samples were collected from different clinical sources: burns, wounds, ear infections, and urinary tract infections. The samples were transferred to the Department of Biotechnology, College of Science, University of Baghdad, to carry out isolation experiments and other tests.

Isolation and identification of *Pseu*domonas spp.: The collected samples were inoculated on blood agar, Maconkey agar, and ceramide agar and incubated at 37ºC for 24 hours. These isolates were identified depending on Gram stain, the cultural characteristics of the colonies, pigment production on ceramide agar, the catalase and oxidase tests, the Simmon citrate test, the starch lysis test, the motility test, the Indole test, the Methyl Red (MR) test, the Voges-Proskaur test, the growth at 4^{o} C and 42^{o} C and finally, the determination of the vitek2 compact system and the *16S rRNA* gene by molecular detection (15).

Pyocyanin production assay: Clinical isolates were cultured on King A and Cetrimide agar medium and then incubated at 37 °C for 48 h. Blue-green pigment formation indicated pyocyanin production ability (33).

Protease enzyme production: The proteolysis of the casein test was performed using skimmed milk agar (28 g skimmed milk powder, 1 g dextrose, 5 g tryptone, 2.5 g yeast extract, and 15 g agar per 1 L, pH 7.0) to evaluate the potential of *P. aeruginosa* isolates to produce protease enzymes. The isolates were spotted on the plates and then incubated at 28 °C for 48 hours. Clear zones were surrounded by the positive isolate's colony (11).

Haemolytic activity: Haemolysis was regarded as a method for biosurfactant synthesis since biosurfactants could potentially induce erythrocyte lysis. *P. aeruginosa* isolates were streaked on blood agar plates. The development of a clear region around the colonies after 48 hours of incubation at 28°C suggested the presence of microorganisms that produce biosurfactants (1).

Antibiotic susceptibility testing: The Kirby Bauer method was subordinated to carry out the susceptibility test for 11 different antibiotic discs, while the colistin antibiotic was measured by the minimum inhibitory concentration (MIC) method (Table 1) as described by (28). All the identified *P. aeruginosa* isolates were tested for antibiotic sensitivity, and the interpretation of inhibition zones was carried out based on the manufacturers' guidelines (8).

Table 1. The antibiotics used in this study

Antibiotic	Symbol	concentration
Piperacillin / tazobactam	TZP	100/10 µg
Ceftazidime	CAZ	30 µg
Piperacillin	PIP	100 µg
Aztreonam	AZT	30 µg
Imipenem	IPM	10 µg
Meropenem	MEM	10 µg
Netilmicin	NET	30 µg
Gentamicin	GEN	10 µg
Tobramycin	TOB	10 µg
Levofloxacin	LVX	5 µg
Ofloxacin	OFX	5 µg
Colistin	CST	360 mg

Molecular study

DNA Extraction: *P. aeruginosa* genomic DNA was extracted using the Genomic DNA Kit, NEB* (England). DNA concentration and purity were determined by the NAS-99 nanodrop spectrophotometer. The determination of DNA concentration was performed by placing 1µl of DNA on a nanodrop spectrophotometer, and the result appeared on the computer. An elution buffer was used as a blank solution.

PCR amplification: The PCR method was used to confirm *P. aeruginosa* identification using the *16SrRNA* gene and detect biofilm production association genes using specific primers (Table 2). The specific primers amplified the regions of 956 bp, 490 bp, and 325 bp of *16SrRNA, phzM and phzS* genes, respectively. These primers were utilized in a 25 µl reaction solution containing 12.5 µl of Master Mix, 1.5 µl of each primer (forward and reverse), 4.5 µl of nuclease-free water, and 5 µl of the purified DNA template. DNA purity and concentration were determined using Nanodrop; the purity ranged from (1.6 to 2.1) and the concentration ranged from 40 ng/µl to 202 ng/µl. The program used was illustrated in Table 2. The PCR products were analysed on the agarose gel electrophoresis (1.5% w/v), formed from 1X TAE buffer with 4 µl of red safe added as a DNA staining dye. The wells were loaded with PCR products (10µl), except for the first well loaded with a

10µl DNA ladder (100–1200 bp) from Biolab Company USA. Then powered at 85 v/mAmp for 70 minutes. After that, the stained bands were displayed under the UV transilluminator (21).

Statistical analysis: The statistical Packages of Social Sciences-SPSS (2019) program was used to detect the effect of different factors on study parameters. The chi-square test was used to compare the significance between percentages (0.05 and 0.01 probability) in this study (13). P-value ≤ 0.05 was considered statistically significant, and P-value ≤ 0.01 was considered highly significant.

Table 2. The primers used in this study (from Macrogen® /Korea).

No	The gene		Primer sequence 5′→3′	Product size (bp)	The reference of the sequence
1	*16SrRNA*	F	GGGGGATCTTCGGACCTCA	956	(32)
		R	TCCTTAGAGTGCCCACCCG		
2	*phzM*	F	CGAAGACTTCTACAGCTACC	325	Designated in this study
		R	GTAGATATCGCCGTTGGAC		
3	*phzS*	F	CGTCGGCATCAATATCCA	490	
		R	GACGATCATGGTCTTGCC		

PCR program for amplification of *Pseudomonas aeruginosa* genes

Steps	Temperature	Time (m : s)	Number of Cycle
Initial Denaturation	95°C	03:00	1
Denaturation	95°C	00:30	35
Annealing *16S rRNA*	58°C	00:45	
Annealing *phzS*	49°C	00:45	
Annealing *phzM*	49°C	00:45	
Extension	72°C	00:30	
Final extension	72°C	07:00	1
Hold	4°C	10:00	

RESULTS AND DISCUSSION

Isolation of *Pseudomonas* spp.

Two hundred forty bacterial isolates were obtained from different clinical sources: burns, wounds, urinary tract infections, and ear infections. Only one hundred forty isolates were identified as *P. aeruginosa* by morphological characteristics and biochemical tests. The isolates appeared to be a large, round, and irregular bacterium that is bluish green in color and smells like grapes (10). And positive for oxidase, catalase tests, Simon citrate, and motility, while negative for Gram stain, indole, Voges-Proskauer, methyl red, and starch hydrolysis (14, 23). The Vitek 2 compact system test proved that the 140 isolates were *P. aeruginosa*, with a probability ranging between 91 and 99 percent, and the results of PCR confirm these results. The results of PCR exhibited that the gene *16S rRNA* has a volume of 956 base pairs when comparing the multiplication packets with the volumetric guide (DNA ladder), and it was observed that the size of the packages was like the expected size (34, 38) (Figure 1).

Figure 1. Agarose gel electrophoresis (1.5% agarose, 85V/cm2 for 70 min) of *16S rRNA* gene (956bp) of *Pseudomonas aeruginosa*

Detection of some virulence factors

Hemolysin production: The current study results showed that 100% of *P. aeruginosa* isolates produced hemolysin (Figure 1-A and Table 3). Many studies reported the same results for hemolysin production by their *P. aeruginosa* isolates (3).

Protease production: The protease enzyme production assay result (Table 3) showed that 80% of the bacterial isolates produced protease enzymes on the skimmed milk agar (Figure 1-B). These results are consistent with the results of (2,3) which mentioned that 58.57% and 80.1%, respectively, of *P. aeruginosa* isolates produced protease enzymes.

Pyocyanin production: Pyocyanin production is considered a marker of virulence. Table 3 showed that 72.15% of the isolates produced pyocyanin. Pyocyanin-producing *P. aeruginosa* isolates were assessed through the phenotypic diagnosis of the pyocyanin dye synthesis (Figure 1-C). The present study declared that out of 140 *P. aeruginosa* isolates, 101 (72.15) produced pyocyanin, of which 37 formed a dark-colored pyocyanin (high), 64 formed a light-colored pyocyanin (moderate), and 39 were non-producers (non). The moderate pyocyanin producers recorded a significantly higher percentage than the non- and high producers (Table 4). The differences in pyocyanin production might be due to the presence of the genes responsible for the production of pyocyanin and their ability to be expressed. This result is consistent with a previous report by (32). The isolates that produce blue-green pigment (pyocyanin) were more resistant to antibiotics than the isolates that did not.

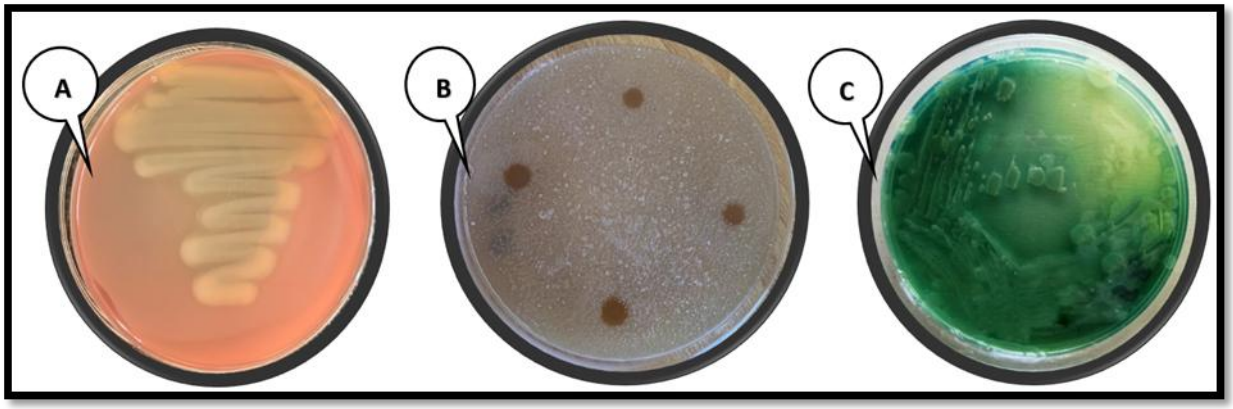


Figure 2. *Pseudomonas aeruginosa* growth on (A) Blood agar to show hemolysin production, (B) skimmed milk agar to show protease production, and (C) King A medium to show pyocyanin production

Table 3. The percentage of *Pseudomonas aeruginosa* isolates capable of producing some virulence factors.

Virulence factor	Positive results	Negative results
Hemolysin	100%	0%
Protease	80%	20%
Pyocyanin	72.15%	27.85%

***The results are for 140 isolates**

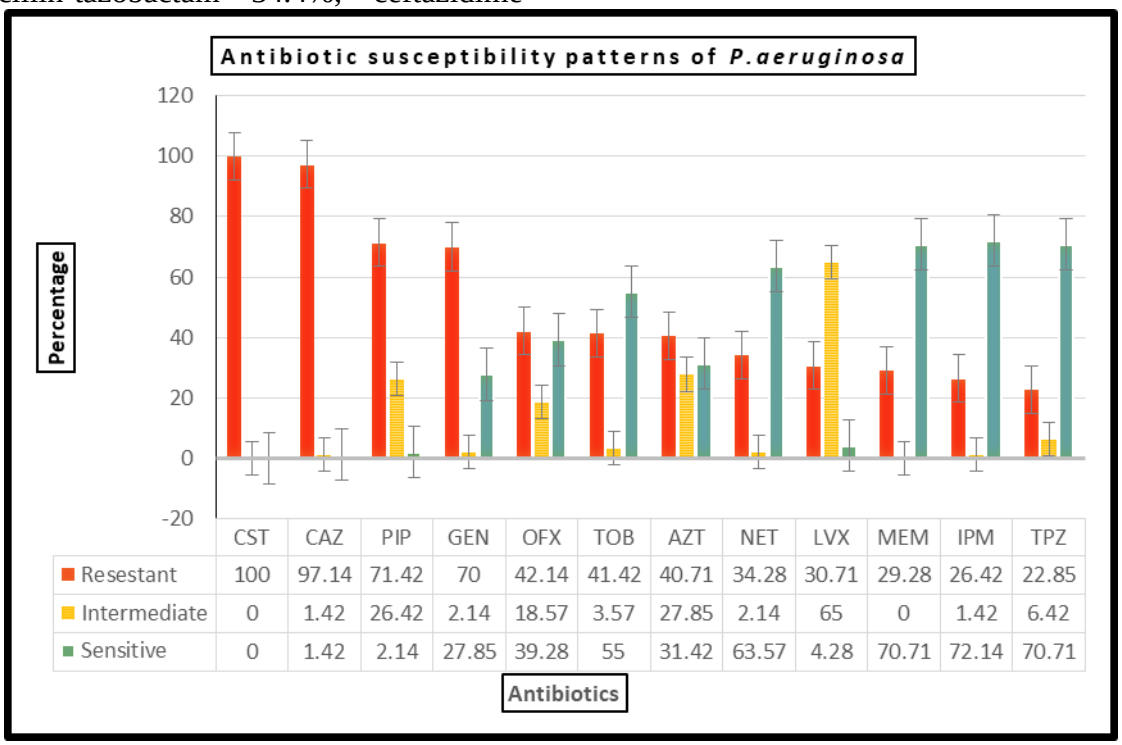
Table 4. The pyocyanin production ability of *Pseudomonas aeruginosa* isolates

Pyocyanin productivity	Number of isolates	Percentage
Non	39	27.85 %
Moderate	64	45.72 %
strong	37	26.43 %
Chi-Square (χ^2)	---	9.812 **
P-value	---	0.0074
** P≤0.01.		

Antibiotic susceptibility test

The antibiotic susceptibility results of 140 *P. aeruginosa* bacterial isolates (Figure 3) showed high resistance to colistin (100%), ceftazidime (97.14%), piperacillin (71.42%), gentamicin (70%) and low resistance to meropenem (29.28%), imipenem (26.42%), and piperacillin-tazobactam (22.85%). Through the statistical analysis of this result we found the P-values for all the antibiotics

were highly significant (0.0001 except for OFX, which is 0.0009), except for AZT, which is non-significant (0.228).This study is consistent with a previous report by (16), which demonstrated that *P. aeruginosa* isolates resistance was 72.22% to piperacillin, 47.22% to aztreonam, 45.83% to tobramycin, 44.44% to meropenem, 40.28% to imipenem, and 33.33% to levofloxacin, but inconsistent with the same study in regard to ceftazidime 68%, gentamycin 51.39%, and colistin 40.28%. While contradicts with studies that showed less resistance to Ceftazidime, Aztronam, Imipenem, and Aztronem (9, 27). Also inconsistent with the study of (19), who mentioned that the resistance percentages of *P. aeruginosa* were for imipenem 60%, meropenem 53.7%, aztreonam 44.4%, piperacillin 43.1%, levofloxacin 40.0%, piperacillin-tazobactam 34.4%, ceftazidime 30.0%, gentamicin 11.9%, and Tobramycin 5%. (15) found that the resistance of *P. aeruginosa* isolates to ceftazidime was 90.5%, which was convergent with our study, and to gentamicin was 88.5%, which disagreed with our study. The results of testing the activity of twelve different antibiotics from eight different groups according to (8). The ability of the isolates to produce pyocyanin was compared with that of the isolated *P. aeruginosa*. The comparison revealed that the isolates with no pyocyanin productivity were resistant to (2–7) antibiotics, the isolates with moderate productivity were resistant to (4–8) antibiotics, and the isolates with high productivity were resistant to (5–12) antibiotics. Indicating that the isolates with a higher percentage of pyocyanin productivity were more resistant.



	CST	CAZ	PIP	GEN	OFX	TOB	AZT	NET	LVX	MEM	IPM	TPZ
Resestant	100	97.14	71.42	70	42.14	41.42	40.71	34.28	30.71	29.28	26.42	22.85
Intermediate	0	1.42	26.42	2.14	18.57	3.57	27.85	2.14	65	0	1.42	6.42
Sensitive	0	1.42	2.14	27.85	39.28	55	31.42	63.57	4.28	70.71	72.14	70.71

Figure 3. The percentage of antibiotic susceptibility for *Pseudomonas aeruginosa* isolates. The P-value was highly significant (0.0001) for resistant, intermediate, and sensitive (P≤0.01).

Detection of *phzS* and *phzM* genes

For detecting the *phzS* and *phzM* genes, sixty *P. aeruginosa* isolates with varying levels of pyocyanin productivity and antibiotic resistance were chosen. The studied *P. aeruginosa* isolates were subjected to PCR amplification to detect the presence of the *phzS* and *phzM* genes that are responsible for pyocyanin production. The result of gel electrophoresis for the amplification PCR product revealed that the appearance of the *phzS* and *phzM* genes in the isolates was highly significant, while the appearance of the *phzS* (90%) was significantly higher than *phzM* (70%), as shown in (Figure 4) and (Table 5).

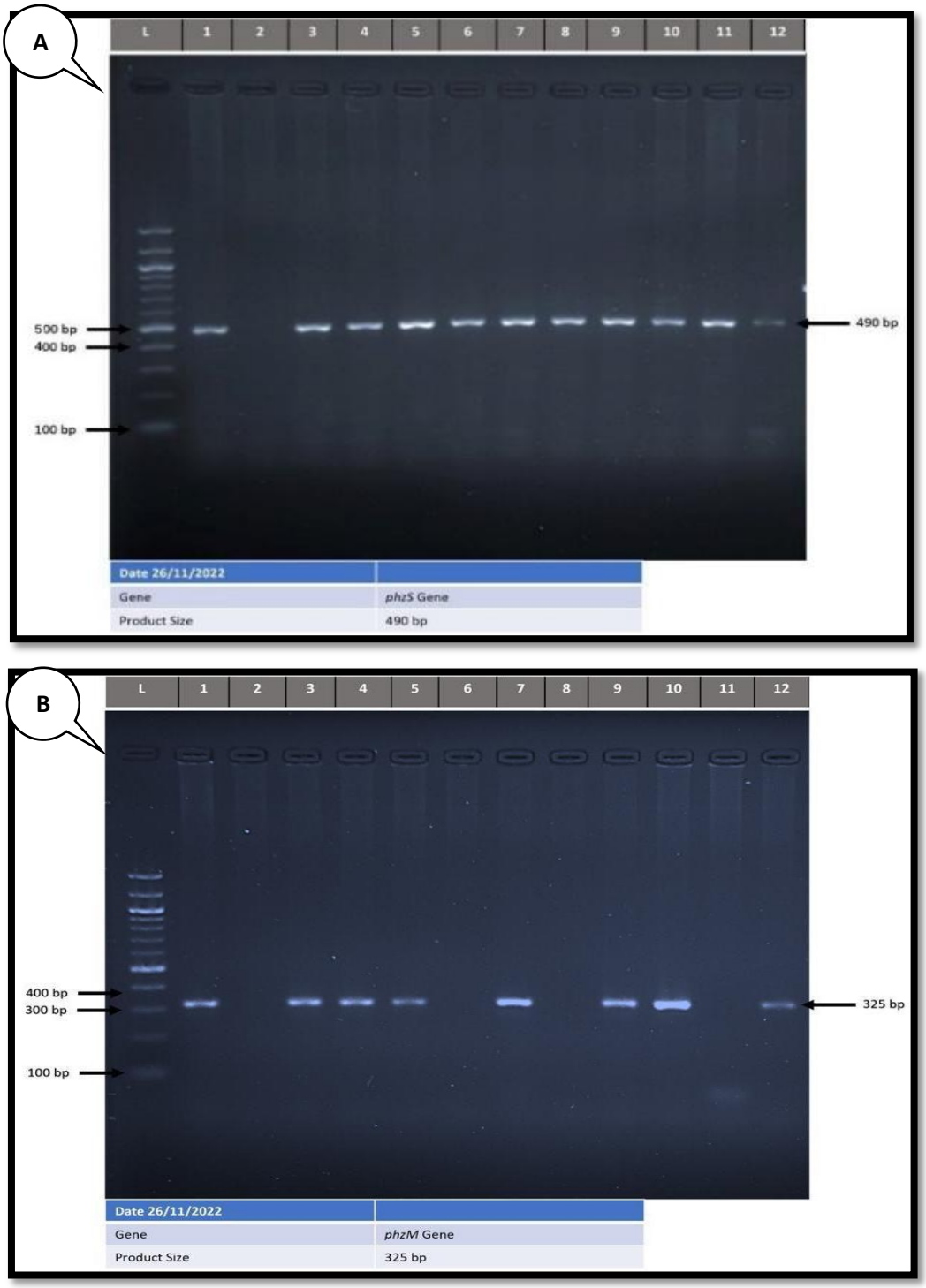


Figure 4. *Pseudomonas aeruginosa* detected genes in 1X-TAE buffer and 1.5% Agarose gel, (A) *phzS* (490bp) and (B) *phzM* (325bp).

Table 5. The percentage of *phzS and phzM* genes responsible for pyocyanin production by *Pseudomonas aeruginosa* isolates

Genes	+ve No (%)	-ve No (%)	Chi-Square (χ^2) (P-value)
16SrRNA	60 (100%)	0 (0.00%)	23.071 ** (0.0001)
phzS	54 (90.00)%	6 (10.00%)	18.592 ** (0.0001)
phzM	42 (70.00%)	18 (30.00%)	12.744 ** (0.0001)

** (P≤0.01).

The isolates that show the presence of both *phzS* and *phzM* genes were either moderate or strong pyocyanin producers, which were 20 (33.33%) or 22 (36.66%) isolates respectively. Meanwhile, the non-producing pyocyanin isolates either showed the absence of both *phzS* and *phzM* genes or only the *phzS* gene, which were 6 (10%) and 12 (20%), respectively. (Table 6). The incidence of both *phz*S and *phz*M genes in *P. aeruginosa* is necessary for their ability to produce pyocyanin. This result is consistent with a previous reports by (20, 32). On the other hand the isolates with high pyocyanin productivity show high resistance to antibiotics. Previous research by (17) mentioned that *P. aeruginosa* isolates that do not produce pyocyanin have low pathogenicity and lower resistance to antibiotics.

Table 6. The relation between pyocyanin production and the appearance of the *phzS* and *phzM* genes in *Pseudomonas aeruginosa* isolates

percentage (number) of the isolates	Pyocyanin production	Appearance *phzS* gene	Appearance *phzM* gene
10% (6)	Non	-	-
20% (12)	Non	+	-
36.66% (22)	Moderate	+	+
33.33% (20)	Strong	+	+

(-) The gene absence, (+) the gene presence

Conclusion

Pseudomonas species were prevalent in various infection sources (burns, wounds, middle ear infections, and urinary tract infections). Among them, *Pseudomonas aeruginosa* was the most prevalent, particularly in hospital settings, with a notable association with burn wounds and urinary tract infections. The majority of *P. aeruginosa* were pyocyanin and protease producers, and all were hemolysin producers. High levels of antibiotic resistance were observed among *P. aeruginosa* isolates, especially against colistin and ceftazidime, which may reflect the overuse of these antibiotics in managing multidrug-resistant (MDR) infections. In contrast, the lowest resistance rates were recorded for imipenem and piperacillin–tazobactam. Additionally, isolates with higher pyocyanin production tended to show increased resistance to antibiotics. The *phzS* gene was more frequently detected than the *phzM* gene among *P. aeruginosa* isolates. Both genes are essential for pyocyanin production; the absence of either gene results in a lack of pyocyanin synthesis. These findings highlight the urgent need to strengthen antibiotic stewardship and implement effective strategies to limit the spread of resistant isolates and reduce the risk of hospital-acquired infections.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

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