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Carbapenem Resistance Related with Phenazine Genes in Clinical *Pseudomonas aeruginosa* Isolates

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ABSTRACT
Background: *Pseudomonas aeruginosa* is a commonly known opportunistic bacteria that can produce a wide range of biologically active compounds. The ability of certain strains to generate extra pigments such as yellow pyoverdin, dark red pyorubin, and pyomelanin (dark black), as well as the two primary dyes, were pyocyanin (blue-green) and fluorescein (yellow-green). **Aims:** To investigate the frequency of some virulence factors genes (phz M, *phz S)*, of carbapenem resistance *P. aeruginosa* local isolates.
Methods: Clinical samples were taken from wounds, burns, urine, nose, and ear swabs, and 110 isolates of *Pseudomonas aeruginosa* were obtained from two hundred and ten samples gathered from affected patients, and diffusion method was utilized to assess the resistance of the bacterial isolates. Also was used molecular detection of (*Phz M, Phz S*) genes by using PCR technique. **Results**: Most of the isolates (40) % were found in burn samples whereas in wounds samples (16.3)%, urine (23.7)%, and ear swabs (20)%. In the present study, a prevalent large percentage of *P. aeruginosa* isolates exhibited high levels of resistance to meropenem (20)% and the lowest level to Imipenem (5.5)%. Molecular analysis showed the phz M and phz S genes identified in all of the carbapenem resistance *P.aeruginosa.*
Conclusions: Large percentage of *P. aeruginosa* isolates exhibited high levels of resistance to meropenem (20%) and the lowest percentage to Imipenem (5.5%). The results showed that all isolates have phenazine genes (*Phz S, Phz M*).
Keywords: Carbapenem Resistance, Genotype Distribution, Phenazine Pigments, *Pseudomonas aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is a commonly known opportunistic bacteria that can produce a wide range of biologically active compounds. Certain strains can be able to generate extra pigments such as yellow pyoverdin, dark red pyorubin, and pyomelanin (dark black), as well as the two primary dyes were pyocyanin (blue-green) and fluorescein (yellow-green) [(1)]. *Pseudomonas aeruginosa* produces significantly many compounds which support their pathogenicity, phenazine is this one class of compound, with pyocyanin, the molecule produced from the substrate shikimic acid [(2)].

The creation of blue pus is one of the most focused keys of essential infections caused by this bacteria [(3)]. Strains that are unable to produce pyocyanin are less pathogenic and more susceptible to the immune system response [(4)]. The *phz* gene operons produce phenazine-1-carboxylate (PCA) and two particular genes, *phz M* and *phz S*, that produce pyocyanin[(5)].

Carbapenem antibiotics, including imipenem and meropenem, are potent against infections caused by *P. aeruginosa* [(6)]. *Pseudomonas aeruginosa* develops resistance to carbapenems through a variety of mechanisms, such as the defect outer membrane proteins, production of β-lactamase, overexpression of efflux pumps, the emergence of chromosomal AmpC beta-lactamase gene, might acquire resistance to carbapenems in *P. aeruginosa*[(7)]. As result, presently would get more challenging to treat [(8)].

METHODS

Sample collection and isolation protocol :

Clinical samples were taken from wounds, burns, urine, nose, and ear swabs, and 110 isolates of *Pseudomonas aeruginosa* were obtained from two hundred and ten samples gathered from affected patients from National Center for Educational Laboratories Specialized Burns Hospital, Al-Ramadi Teaching Hospital, during the period from November 2021 to February 2022. Microscopical, biochemical, and cultural characteristics are used to identify and classify *Pseudomonas aeruginosa.*

Antimicrobial susceptibility test :

Diffusion method was utilized to assess the resistance of the bacterial isolates according to CLSI-2021): A few colonies (2-4) were added in (2 ml) brain-heart broth and cultured to obtain a bacterial suspension with a McFarland turbidity, A cotton swab was used to culturing the bacterial suspension into Muller Hinton agar plates. Anti-microbial discs were positioned upon the medium's surface and incubated for 24 hours at 37°C. The diameter of each antibiotic disk's inhibitory zone was measured and compared to the CLSI-2021 standard inhibition zone.

Molecular identification:

Molecular detection of (*Phz M*, *Phz S*) genes by using PCR technique and Bioneer kit.

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Table (1): PCR primers and molecular size of the PCR products

Gene	Primers' Sequences	Product size(bp.)	Annealing Tem.
Phz S	**F: CGCCATGACCGATACGCTC**	**1752**	**58**
	R: CAACCTGAGCCAGCCTTCC		
Phz M	**F: TGGAGAGCGGGATCGACAG**	**875**	**58**
	R: ATGCGGGTTTCCATCGGCAG		

F=Forward sequence, R=Reverse sequence.

Preparation of PCR mixture :

The master mix by using a Bioneer kit, primer solution, deionized water, and template DNA was combined in 20 µl of PCR reaction, with the PCR mixture used in the study**.**

***16SrRNA* gene detection using PCR program:**

Table (2): *16SrRNA* gene detection PCR amplification program.

Step	Temp. (C^{o})	Time (min)	No. of cycle
Initial denaturation	**95**	**5**	**1**
Denaturation	**94**	**1**	**30**
Annealing	**58**	**1**	
Extension	**72**	**1**	**30**
Final extension	**72**	**7**	**1**
Hold Temperature	**4**	**4**	**-**

While the main product was stored at 20^{o}C, 2 microliters of it were electrophoresed.

Table (3): Polymerase chain reaction conditions for the phz M gene.

Gene	Step	Temperature (^{0}C)	Time (min.)	No. of cycle
	Initial denaturation	**95**	**5**	**1**
	Denaturation	**95**	**30 s.**	**30**
	Annealing	**58**	**30 s.**	**30**
	Extension	**72**	**1 min**	
phz M	**Final extension**	**72**	**7 min.**	**1**
	Hold temperature	**4**	**4 min**	

Table (4): Conditions for phz S gene polymerase chain reactions.

Gene	Step	Temperature (^{o}C)	Time (min.)	No. of cycle
	Denaturation at the start	**95**	5	1
	Denaturation	**95**	30 s.	30
	Annealing	**58**	30 s.	30
	Extension	**72**	1 min	
phz S	Final extension	**72**	7 min.	**1**
	Hold temperature	4	4 min	

RESULTS AND DISCUSSION

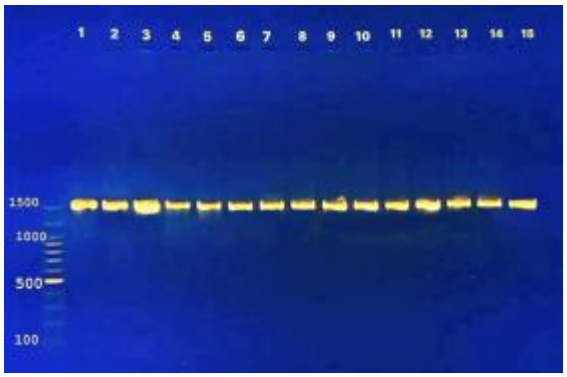
One hundred and ten isolates were confirmed as *P.aeruginosa* out of a total of 210 specimens (burn, wound, ear, and UTI). The study findings revealed that most of the isolates (40%) were found in burn samples. whereas the percentage of isolates from wound samples was (16.3%) isolates, these results agreed with [9] who showed that *P.aeruginosa* from burn samples contained the most proportion of isolates (46.8%), while the wound samples contained the greatest proportion of isolates (31.6%). *Pseudomonas aeruginosa* is the most common pathogen responsible for nosocomial infections in burn patients, it was recognized as a critical burn wound colonizer. Due to its ability to grow on moist burn wound areas of skin and remain alive in hospital environments [10]. Whereas the proportion of other infections is (20& 23.7)% from the ear, and urine swabs, this result differed from the result of **Finlayson *et al* [11],** the isolates were obtained from urine samples (6 or 10.3)% and ear swabs (11 or 19.3) %.

A large percentage of *P. aeruginosa* isolates showed high levels of resistance to meropenem (20%), and the lowest percentage to imipenem (5.5%). The percentage obtained by **Shilba** agrees with our results [12].

Other studies showed different results that the imipenem had a higher percentage [13, 14]. *Pseudomonas aeruginosa* can resist several antibiotics, which can be obtained naturally through mutants in genetic material such as horizontal gene transfer [15]. As indicated in a previous study that mortality was greater in the group of the carbapenem-resistant, but

there was an important correlation between the appearance of resistant strains and inappropriate antimicrobial therapy [16].

16SrRNA gene was used to identify *Pseudomonas aeruginosa* isolates this gene plays a crucial role in diagnosis, in this study, all isolates exhibit the *16S rRNA* gene, **Figure (1**).



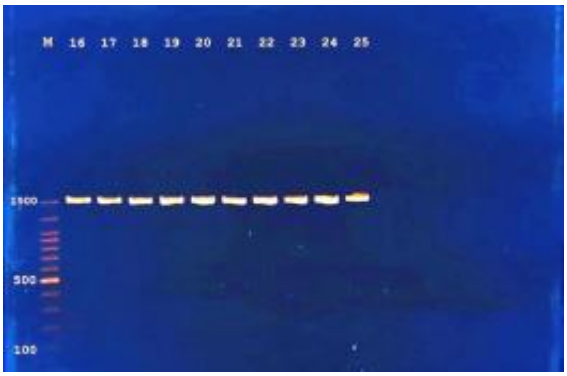
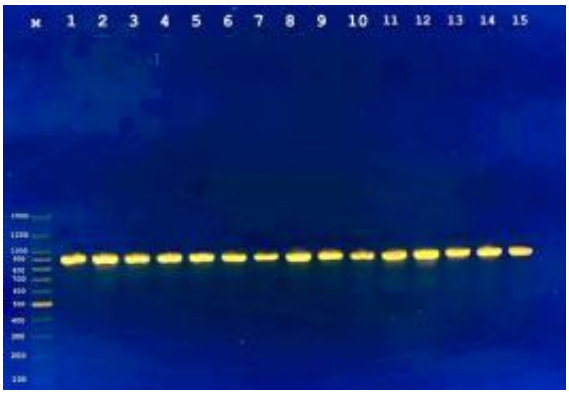


Figure (1): Electrophoresis of the *16srRNA* gene (1500bp),(1.3 % agarose, 70 V/cm^2 - 90min) lane M (100_1500bp) DNA ladder, lanes 1-25 *16SrRNA* represent bands of carbapenem resistance *P. aeruginosa* isolates.

Molecular analysis of 25 isolates showed that the percentage of the *phzM* and *phzS* genes were 100% of the genes responsible for converting PCA to the pyocyanin pigment, **Figures (2)**

The products of the *phz M* gene (875bp) and the *phz S* gene (1752bp) were confirmed by using electrophoresis, as displayed in **Figures (3).**

The finding was consistent with the study showing the two genes noted in all of the carbapenem resistance *P.aeruginosa*, this could indicate that one of the most common virulence factors of CRPA is pyocyanin [17]. But fuse demonstrated that the production of pyocyanin decreases in a group of MDR *P. aeruginosa* strains, according to the findings of our study, all of the CRPA strains carry the *phz M* and *phz S* genes, so it may be the reduced synthesis of pyocyanin related to the resistance to other antibiotic classes, or it may be due to product is more likely regulated at the expression level, not the carriage itself [18]. *Pseudomonas aeruginosa* isolates lacking pyocyanin are less pathogenic and more susceptible to the immune response [19].



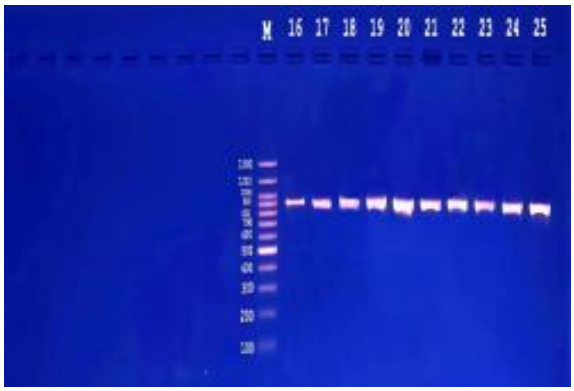
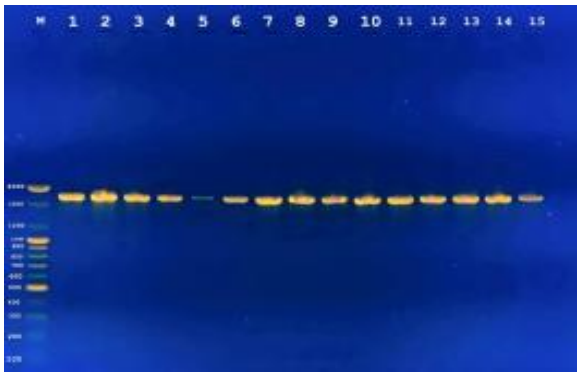


Figure (2): Electrophoresis of the *phz M* gene (875bp), (1.2 % (agarose 70 V/cm^2 for 90 min), lane M represents(100_ 1500bp) DNA ladder, lanes 1-25 represent *phz M* bands of carbapenem resistance *P. aeruginosa* isolates.



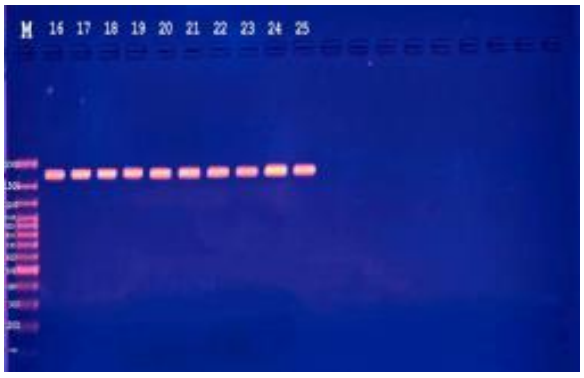


Figure (3): Electrophoresis for the *phz S* gene (1752bp) (1 % agarose, 70 V/cm^2 for 90 min), lane M represents (100_2000bp) DNA ladder, lanes 1-25 represent *phz S* bands of carbapenem resistance *P. aeruginosa* isolates.

CONCLUSIONS

In the current study, a large percentage of *P. aeruginosa* isolates exhibited high levels of resistance to meropenem (20%) and the lowest percentage to Imipenem (5.5%). The results showed that a11 isolates have phenazine genes (*Phz S, Phz M*).

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