

Isolation of *Pseudomonas Aeruginosa* and Studying their Resistance and Pyocyanin Production.

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Abstract

The study included isolating and diagnosing *Pseudomonas Aeruginosa* bacteria from different clinical samples from hospitals and health centers: 10 wound samples, 15 burn samples, 12 ear samples (otitis media), 11 urine samples and 9 sputum samples. These samples were diagnosed using cultural and biochemical features and confirmed by using the API 20E system test. The results showed that the isolation rate of *Pseudomonas Aeruginosa* bacteria from burns was (26.3%) and from ears was (21%). The resistance of *Pseudomonas Aeruginosa* isolates to 6 types of antibiotics was tested. The highest resistance rate (29.8%) was observed against Cefazoline and Carbenicillin, followed by resistance to Cefotaxime (17.5%), then to Gentamicin (14%), followed by Ciprofloxacin and Amikacin, (5.2%) and (3.5%), respectively. The results demonstrated that 18(31.5%) of *Pseudomonas Aeruginosa* isolates produced the pyocyanin pigment on Nutrient agar media and 9(15.7%) of them produced the pigment on MacConkey agar, while 15(26.3%) isolates produced the pigment on Blood agar. The burn isolates were shown to produce the highest pyocyanin pigments, and nutrient agar was the highest media that produced pyocyanin.

1. Introduction:

Pseudomonas Aeruginosa is a gram-negative bacteria belonging to the Pseudomonadaceae family. It is obligate aerobic, motile, and the term Pseudomonad is precisely used to describe aerobic non-spore forming Gram-negative bacteria [1] [2].

Pseudomonas Aeruginosa is an opportunistic pathogenic bacteria that is widely spread in nature and is pathogenic to humans, plants and animals. This bacterium can live in diverse environments. It is free-living and lives in marine marshes, river water, soil and coastal areas [3]. It poses a real danger to hospitalized patients, especially people with burns, cancer patients, organ transplant patients and immunodeficiency patients. It is one of the most important bacterial species that causes nosocomial infections [4]. This bacteria inhabits oper-

ating theaters, hospital wards, surgical instruments, etc. It can also survive in volatile substances and in some disinfectants, and is able to infect many tissues, causing acute and chronic infections to burnt victims as well as to immunocompromised people and those suffering from cystic fibrosis. The wide spectrum of diseases caused by this bacteria depends on its possession of many virulence factors [5].

Pseudomonas Aeruginosa produces pyocyanin pigment, (with a bluish-green color) and its chemical composition is Methylene-1 hydroxy phenazine, which destroys the cilia in the respiratory system [6]. Pyocyanin is produced during the metabolism process with a bluish-green color in water, and excreted outside the cell. Some species of *Pseudomonas Aeruginosa* produce the pigment when grown on appropriate culture media. The ability of this pyocyanin to appear increases when incubated at (28-30°C) since it is a broad-spectrum inhibitor of bacteria [7] [8].

The widespread use of antibiotics to treat these bacteria led to antibiotic resistance development, which may be the reason for plasmid possession by this bacteria, as new strains emerged as more resistant to antibiotics and difficult to treat

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[9] [10].

This bacterium has different resistance mechanisms, the most important of which are changing the target location, changing the permeability of the outer membrane, and producing enzymes that destroy antibiotics, such as β -lactamases, which make it resistant to Penicillins and Cephalosporins. The spread of this bacterium in different parts of the body, and its continuous exposure to antibiotics led to emergence of new strains, which are characterized by multi-drug resistance [11] [12]. Therefore, the current study aimed to isolate *Pseudomonas Aeruginosa* from several clinical sources, study the resistance of *Pseudomonas Aeruginosa* to some antibiotics, identify isolates with multi-drug resistance, determine the number of pyocyanin-producing isolates and determine the effect of the pigment on the growth of pathogenic bacteria species.

2. Methodology:

Clinical sample collection: Clinical specimens were collected from patients who attended to different hospitals and health centers in Baghdad city. Samples were taken from urinary tract infections (urine was collected in clean and sterile containers), wound swabs, burn swabs, ear swabs and sputum (swabs were taken using sterile swabs).

2.1 Bacterial Identification:

Accurate identification of bacterial species: A number of tests and examinations were performed to ensure the purity of the isolates, based on cultural morphologic, microscopic and biochemical examinations such as oxidase, catalase and IMVIC tests, and the diagnosis was confirmed using the API20E system [13], [14].

2.2 Antibiotic Resistance:

The agar diffusion method was used according to the method of [15], and modified by the World Health Organization [16]. The antibiotics used are listed in Table 5.

2.3 Pyocyanin Production Test:

Pseudomonas Aeruginosa was cultured on nutrient agar, blood agar and MacConkey agar. The plates were then incubated at 37°C for 24 hours. The appearance of a bluish-green color in the culture media indicated positive pyocyanin production [17].

2.4 The Effect of Pyocyanin on other Pathogenic Bacteria Species:

In this study, (4) *Pseudomonas Aeruginosa* isolates were grown on Nutrient agar to produce the dye. After 24 hours incubation, the bacterial cells were scraped with a clean glass

slide and the dishes were exposed to chloroform to kill the remaining cells. After that, some isolates such as *Staphylococcus aureus*, *E.coli* and others were cultured on the surface of the medium, which was exposed to chloroform.

The activity of pyocyanin on these pathogenic bacterial isolates is indicated by inhibition of the growth of these pathogenic isolates, so as to confirm the activity. After 24 hours incubation, a single colony of the *Pseudomonas Aeruginosa* bacteria was cultured in Nutrient broth, then the dye was withdrawn using Millipore filters with (0.22 , 0.45) diameters, then saturated with discs of sterile filter papers with fixed diameters for (3-5) minutes. After that, the other bacteria were cultured on the culture medium used, then the discs were placed using sterile forceps on the cultured dish and incubated for 24 hours, followed by measurement of the diameter of inhibition [18].

3. Results and Discussion:

From different clinical sources, (57) isolates of *Pseudomonas Aeruginosa* were obtained including (burns, wounds, ear and sputum) as shown in Table 2. The results of the current study showed that the highest rate (26.3%) of *Pseudomonas Aeruginosa* was obtained from burns, which coincided with the results obtained by [19], who found that (38%) of the bacterial isolates were obtained from burns, while the study conducted by [1] reported that (67.6%) of this bacterium was isolated from burn samples. The convergence and difference in the isolation rate of the bacterium from the samples may be due to differences in sample size and source. The current study also showed that 10(17.5%) of *Pseudomonas Aeruginosa* isolates were obtained from wounds. This result did not agree with [20], who stated that (94.5%) of the bacterium was isolated from wounds, and also with [21], who isolated only (28.2%) of the bacterium from wound samples. Likewise, we found that 11(19.2%) of the bacterium *Pseudomonas Aeruginosa* was isolated from urine samples, and this result was not in agreement with the results of [19], who isolated (51.1%) of the bacterium from urine samples.

In the current study, urinary tract infections came in the second degree after burn infections, which indicates the role of this bacterium in urinary tract infections, especially in patients who undergo urinary tract catheters, or it may be due to the adhesion factors possessed by this bacterium, which facilitate the process of its adhesion to catheter materials and its resistance to antibiotics [22]. Also, 8 (15.7%) isolates of this bacterium were obtained from the sputum in our study, and this result was expected since this bacterium isolated from sputum is an opportunistic species, and perhaps its ability to infect immunocompromised hospitalized individuals or due to nosocomial infections. Our results reported that 12(21%) isolates were obtained from ear samples, which was not consistent with the study of [23], who isolated (68.9%) of the

bacterium from ear samples, and also with the study of [24], who found that (5.19%) were isolated from ear samples.

Table 1. Prevalence of *P. Aeruginosa* in clinical samples.

Antibiotic/ concentration	Isolate resistance	%
Amikacin 10 mcg	2	3.5
Carbenicillin 100 mcg	17	29.8
Gentamycin 30 mcg	8	14
Cefatoxim 10 mcg		
Sputum	9	15.8
Total	57	100100

3.1 Antibiotic Resistance:

The isolates showed resistance to the antibiotics used in the study including Gentamicin, Cefotaxime, Amikacin, Carbenicillin, Ciprofloxacin, and Cefazoline, and these isolates are known to possess auto and acquired resistance to many widely-used antimicrobial drugs [25]. The presence, concentration, and type of protein channels (porins) located on the outer membrane of the *Pseudomonas* spp., are responsible for the influx mechanism for many compounds, including antibiotics such as beta-lactam antibiotics. There was a variation in the resistance of the isolates of *Pseudomonas Aeruginosa* bacteria. The results showed that the highest resistance rate (29.8%) of the pathogenic isolates was to Cefazoline and Carbenicillin, followed by resistance to Cefotaxime (17.5%) then to Gentamicin (14%) followed by Ciprofloxacin and Amikacin (5.2%) and (3.5%), respectively as shown in Table 2 and Figure 1.

The high resistance of these isolates to some antibiotics Cefazoline, Carbenicillin, and Cefotaxime indicates the possibility of production of β -lactamase enzymes that have a great ability to destroy the β -lactam antibiotic group. Also, the extensive use of antibiotics from this group for a long period in the treatment of different infections resulted in the emergence of strains that produce a wide range of β -lactamase enzymes, which are encoded in plasmids or in chromosomes due to the presence of transposons [26]. In regard with the antibiotic Ciprofloxacin from the Quinolones group, our results were not consistent with the findings of [27], who stated that this antibiotic is effective against *Pseudomonas Aeruginosa* bacteria, and they found a resistance rate of (58.3%), while the resistance in our study was (5.2%). However, it was close to the results obtained by [28], who revealed a resistance rate of (9%), and this may be due to the fact that this antibiotic is one of the newly-used antibiotic, and the bacteria have not developed a mechanism to resist it, and this antibiotic works to inhibit the activity of the DNA gyrase enzyme [29].

As for the aminoglycoside group of antibiotics including Gentamicin and Amikacin, which are considered among the

antibiotics that affect *Pseudomonas Aeruginosa* [30], it has been found that resistance to aminoglycoside antibiotics has been increasing significantly in recent years, and this resistance is due to the production of an enzyme that works to modify the antibiotic, and thus these antibiotics lose their properties or come as a result of the loss of some outer membrane proteins, which reduce the permeability of the antibiotic into the cell. Therefore, these antibiotics have shown variation in terms of their effect on *Pseudomonas Aeruginosa* bacteria [31].

Table 2. Resistance of isolates to antibiotics / percentage.

Antibiotic/ concentration	Isolate resistance	%
Amikacin 10 mcg	2	3.5
Carbenicillin 100 mcg	17	29.8
Gentamycin 30 mcg	8	14
Cefatoxim 10 mcg	10	17.5
Ciprofloxacin 10 mcg	3	5.4
Cefazoline 5 mcg	17	29.8
Total	57	100

3.2 Pyocyanin Production:

The results shown in Figure 2 and Table 3 demonstrated that 18(31.5%) of clinical isolates of *Pseudomonas Aeruginosa* produced pyocyanin pigment on Nutrient agar, 9(15.7%) isolates produced the pigment on MacConkey agar, and 15(26.3%) isolates produced the pigment on Blood agar, and the result is similar to what was found by [15]. The burn isolates were shown to be the highest pyocyanin pigment producing, and Nutrient agar was the highest media that produced pyocyanin.

Table 3. Pyocyanin production by *Pseudomonas Aeruginosa* on different media.

Blood agar	Mac Conkey agar	Muller Hinton agar	Nutrient agar	Medium / Isolates
26.3%	15.7%	31.5%	31.5%	Clinical isolate

3.3 Testing the Sensitivity of Some Bacteria to Pyocyanin Pigment:

The results shown in Figures 3, 4 and Table 4 showed that *Staphylococcus aureus* bacteria was more sensitive when cultured on Nutrient agar and Muller Hinton, which contain pyocyanin pigment, while Gram-negative bacteria such as *Escherichia coli* were resistant to pyocyanin pigment. This result is consistent with the findings of [32, 33] who showed that py-

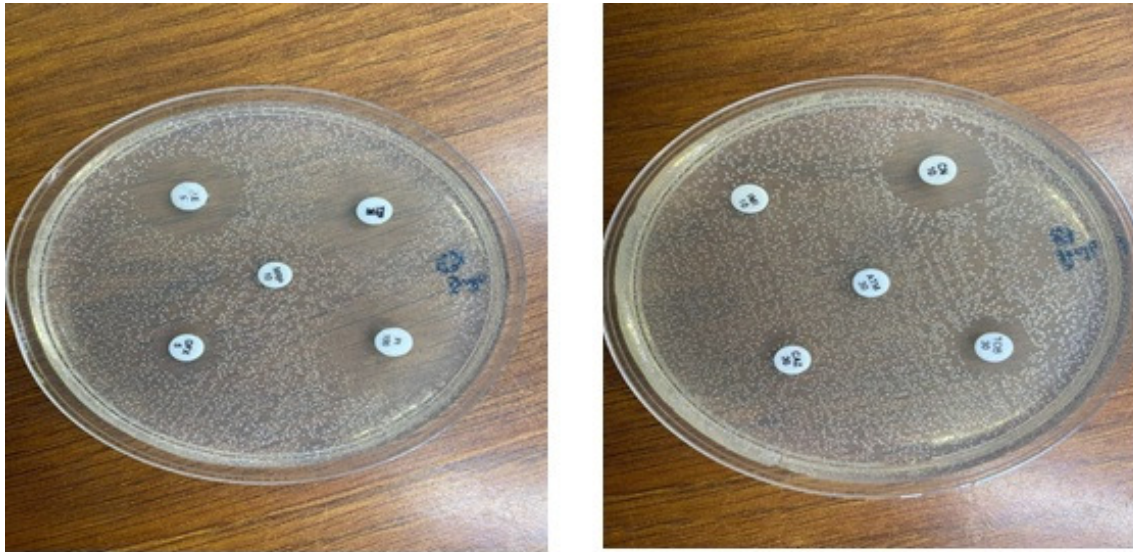


Figure 1. The effect of weights of NaCl on stress-strain curves for CMCHV thin films.

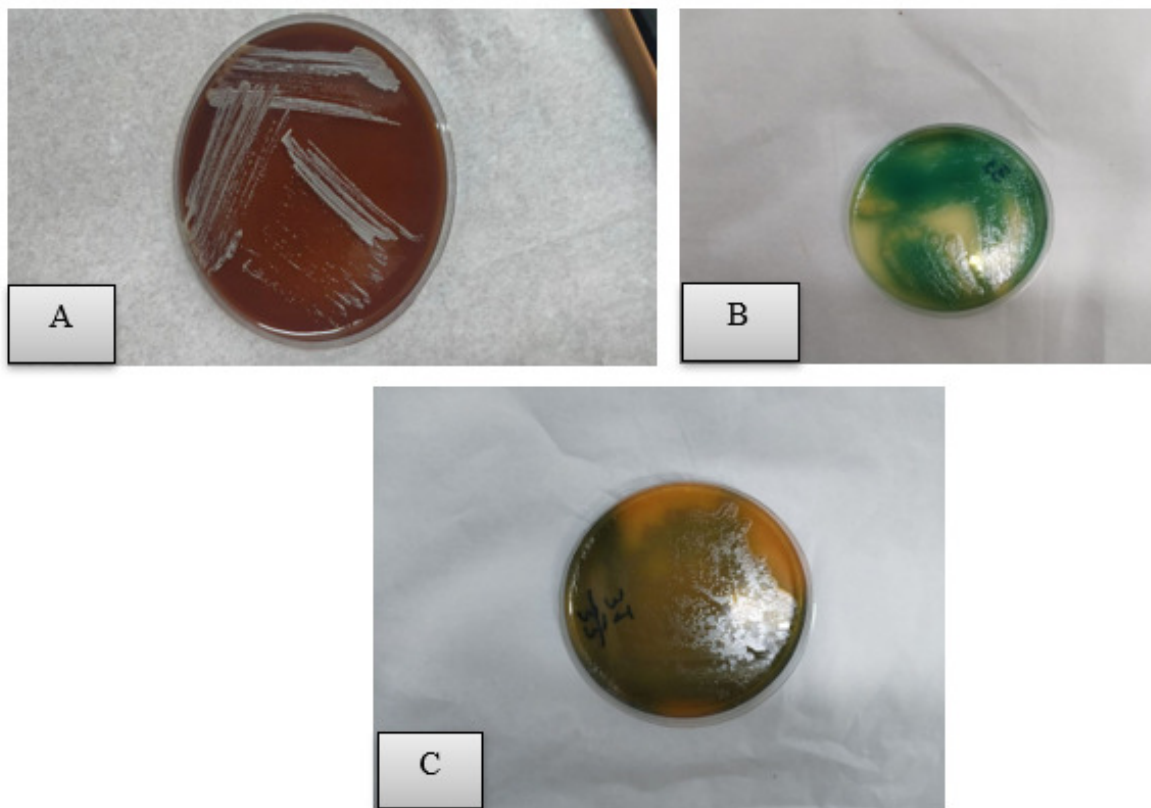


Figure 2. The effect of weights of ZnO NPs on stress-strain curves for CMCHV thin films.

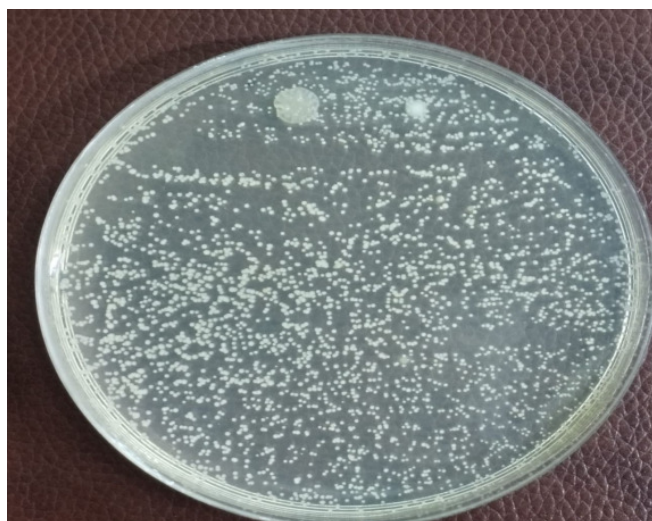


Figure 3. *Staphylococcus aureus* growth on a medium containing pyocyanin pigment.

ocyanin is an active compound that inhibits the growth of all Gram-positive bacteria compared to Gram-negative bacteria.

Table 4. Pyocyanin production by *Pseudomonas Aeruginosa* on different media.

Microorganisms	No. of isolates	Pseud 1	Pseud 2	Pseud 3	Pseud 4
<i>Staphylococcus aureus</i>	6	S	S	S	S
<i>Escherichia coli</i>	6	R	I	R	R

Table 5. Antibiotics used in the study.

Antibiotic	Symbol	Concentration (mcg)
Amikacin	AK	10
Carbenicillin	PY	100
Gentamicin	CN	30
Cefotaxime	CTX	10
Ciprofloxacin	CIP	10
Cefazoline	CZ	5

4. Conclusion:

It can be concluded from our study that the highest rate of *Pseudomonas Aeruginosa* isolated from burns resisted six types of antibiotics compared to other clinical sample isolates.

The results showed the highest percentage of resistance was to Cefazoline and Carbenicillin > Cefotaxime > Gentamicin > Ciprofloxacin and Amikacin. Regarding the production



Figure 4. *E. coli* growth on a medium containing pyocyanin pigment.

of pyocyanin dye, the results showed that 18 isolates of *Pseudomonas Aeruginosa* produced pyocyanin dye on Nutrient agar, 9 isolates produced the dye on MacConkey agar and 15 isolates produced the it on Blood agar. The burn isolates were shown to be the highest pyocyanin pigment producing, and Nutrient agar was the highest media that produced pyocyanin.

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Data Availability Statement: All of the data supporting the findings of the presented study are available from corresponding author on request.

Declarations:

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: The manuscript has not been published or submitted to another journal, nor is it under review.

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عزل بكتيريا *Pseudomonas aeruginosa* ودراسة مقاومتها للمضادات الحيوية وإنتاج البيوسيانين

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الخلاصة

تضمنت الدراسة عزل وتشخيص بكتيريا الزائفة الزنجارية من مصادر سريرية مختلفة من المستشفيات والمراكز الصحية في بغداد وكالاتي: 10 عينات من مصادر سريرية من التهابات الجروح، 15 عينة حروق، 12 عينة من التهاب الأذن الوسطى، 11 من الأذنين و 9 عينات من القشع. وشخصت هذه الحالات باستخدام الفحوصات الزرع والكيموحيوية، وتم التأكيد التشخيصي لها باستخدام اختبار 20E API وبينت النتائج ان اعلى نسبة عزل لبكتيريا الزائفة الزنجارية كانت من الاذن بنسبة 21% والحروق بنسبة 27.3%. فخصت مقاومة عزلات الزائفة الزنجارية تجاه 6 انواع من المضادات الحيوية وأظهرت النتائج أن أعلى نسبة مقاومة كانت ضد المضادين Cefazoline و Carbenicillin وبنسبة (29.8%) تلتها المقاومة للمضاد الحيوي Cefotaxim بنسبة 17.5% ثم المضاد Gentamicin بنسبة 14% تلتها كل من المضادين Ciprofloxacin و Amikacin بنسبة 5.2% و 3.5% على التوالي. وأظهرت النتائج ان 18 عزلة (31.5%) من الزائفة الزنجارية أنتجت صبغة البايوسيانين على الوسط Nutrient و 9 (15.7%) أنتجت الصبغة على وسط MacConkey agar و 15 عزلة (26.3%) على وسط Blood agar ان أكثر العزلات إنتاجاً للصبغة اثناء الدراسة هي عزلات الحروق، بينما كان Nutrient agar هو أعلى الأوساط الزرع إنتاجاً للصبغة البايوسيانين.

الكلمات الدالة: الزائفة الزنجارية؛ العينات السريرية؛ المقاومة؛ صبغة البايوسيانين.

التمويل: لا يوجد.

بيان توفر البيانات: جميع البيانات الداعمة لنتائج الدراسة المقدمة يمكن طلبها من المؤلف المسؤول.

اقرارات:

تضارب المصالح: يقر المؤلفون أنه ليس لديهم تضارب في المصالح.

الموافقة الأخلاقية: لم يتم نشر المخطوطة أو تقديمها لمجلة أخرى، كما أنها ليست قيد المراجعة.