Characterization and Molecular Detection Of *bla$_{IMP}$* Gene From Clinical Isolates Of *Pseudomonas aeruginosa* and Its Implication On Antibiotic Resistance and Biofilm Formation

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ABSTRACT

Background: This research is centered on identifying the *bla$_{IMP}$* gene in samples of *Pseudomonas aeruginosa* providing insights into its impact on resistance and the formation of biofilms by utilizing methods and enhancing persistence and complicating treatment, for infections triggered by this microorganism. **Objectives:** molecular detection of *bla$_{IMP}$* gene, in *Pseudomonas aeruginosa* samples obtained from patients and evaluation of its relationship to resistance against antibiotics and the development of biofilms. Comprehend the significance of the *bla$_{IMP}$* gene in terms of resistance and its impact on biofilm-related persistence. Investigate approaches that can be used to tackle resistance caused by *bla$_{IMP}$* and the formation of biofilms, in case of *Pseudomonas aeruginosa* infections. **Methodology**: Twenty *Pseudomonas aeruginosa* isolates were collected from Al-Nahrain University's College of Biotechnology to test the development of biofilms, identify virulence genes using molecular means, and discover ways to reduce antibiotic resistance. **Results:**The results of biochemical detection of 20 *Pseudomonas aeruginosa* isolates revealed that bacterial isolates were negative for indole while Citrate and catalase were positive and these isolates were pyocyanin-producing and sensitive to imipenem, Doxycycline, Levofloxacin, Cefotaxime, gentamicin, and aztreonam with an inhibition zone of 10,6,2,0,10,8 mm respectively. The results of biofilm formation of selected 20 isolates indicated that 10% weak, 30% moderate, 60% strong, and non-biofilm producer. Also, PCR amplification used to detect bacterial isolates as *Pseudomonas aeruginosa* revealed that bands appeared in amplification by 16S rRNA gene and the *bla IMP* gene. Results of the antibacterial activity of quantum dots nanoparticles on *Pseudomonas aeruginosa* showed that at four concentrations tested (100,75,50 and 25 µg/ml), all tested isolates showed an inhibition zone. Finally, the results of cytotoxicity assays of nanoparticles using MTT assay on the HepG2 liver cancer line revealed.

Keywords: *Pseudomonas aeruginosa*, quantum dots, liver cancer, MTT assay.

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INTRODUCTION

*Pseudomonas aeruginosa*is a bacterium that can adapt to different environments and places, like soil and water is also known for causing infections in humans who are already sick or have weakened immune systems in hospitals, *Pseudomonas aeruginosa*can lead to types of infections such as respiratory tract infections (like pneumonia and lung infections related to cystic fibrosis) wound infections, and even severe bloodstream infections (1) One of these substances is called the IMP gene. This gene makes an enzyme, which is called IMP-type beta-lactamase, and this enzyme can break down antibiotics called beta-lactams. In the presence of the gene and enzyme, *Pseudomonas aeruginosa* can survive the effects of antibiotics, making these infections difficult to treat. Understanding how this opportunistic human pathogen operates will equip us with strategies to fight it when it occurs(2).The *bla $_{IMP}$* gene

found in *Pseudomonas* coding to be deleted is the IMP type beta-lactamase (3)These enzymes play a role in bacteria developing resistance to these antibiotics. The *bla* $_{IMP}$gene belongs to a group of beta-lactamases (MBLs) that require metal ions, and zinc for their enzymatic activity (4) Encoding the IMP-type beta-lactamase enzyme's synthesis in Pseudomonas and other Gram-negative bacteria is its main purpose. The beta-lactam ring in beta-lactam antibiotics can be broken by this enzyme, rendering them useless against the growth of bacteria. (4)

Quantum dots (QDs) are semiconductor nanoparticles with organic protection to enhance stability and biological compatibility, a capping shell, and the metalloid crystalline nucleus (5). QDs have distinct optical characteristics, including high extinction coefficients, broad absorption and narrow emission spectra, and consistent sizes.(6)Moreover, QDs are easy to follow and found in several matrices, in part because of their resilient, continuous fluorescence. QDs able to combine with a broad range of biomolecules, this feature(7), gives researchers in biology and physics novel approaches to bioimaging, solar cells, and pharmaceutical delivery.

This study aimed to detect the virulence factor gene of *Pseudomonas aeruginosa* and its ability to resist antibiotics and how can decrease the virulence factor of bla_{IMP} gene by using nanoparticles after testing the anticancer activity.

METHODOLGY

1-Samples collection.

During the experiment, sterile swabs were to use *Pseudomonas aeruginosa* isolates from the Baghdad Teaching Hospital. Afterward, 20 isolate (ear infections, urinary tract infections, and wound infections).

2-Biochemical tests of *Pseudomonas aeruginosa*.

- Indole Test

A loopful of the Pseudomonas culture was picked and added to a tube of tryptone broth. Then the broth was incubated at a temperature of 37°C for 24 hours. When the incubation period was over, a drop of Kovacs reagent was added to the broth. This reagent reacts with indole, which causes a red or pink color to appear. The tube was shaken and observed for any color changes.

- Catalase test.

A small portion of the Pseudomonas culture is put on a glass slide followed by adding a drop of 3% hydrogen peroxide directly onto the culture and the reaction was observed. If catalase is present, it will trigger the breakdown of hydrogen peroxide and generate oxygen gas bubbles.

- Simmons Citrate test.

As directed by the manufacturer, 2.28 g of Simmons Citrate was added to 100 ml of distilled water for the Simmons Citrate medium test, Confirmation of an outcome, in the Simmons Citrate test indicates that the microorganism under examination carries the citrate permease enzyme for citrate transportation into the cell for metabolic processes along with citrate lyase, for breaking down citrate into pyruvate and other metabolic byproducts.(8).

3-Antibiotics susceptibility testing.

Mueller-Hinton agar plates were used and the disk diffusion method was followed to detect the susceptibility pattern of the isolates to various antibiotics, Imipenem (10µg), ciprofloxacin (5µg), ceftazidime (30µg), amikacin (30µg), gentamicin (10µg), and aztreonam (30µg) (9).

4-Biofilm formation assay

- To evaluate the capacity of *P. aeruginosa* isolates to form biofilm the following steps were conducted. After incubation cultures were diluted to (OD) at a wavelength of 600 nm. Typically, it is around 0.1. Transferred the cultures into the wells of a microtiter plate (96-well plate).Then the plate at 37°C for 24 hours to allow biofilm formation on the surface of the wells.
- Biofilm Staining and Quantification; the culture medium was removed carefully from the wells after that the wells were Gently rinsed with phosphate-buffered saline (PBS) to eliminate adherent cells after that Allowed time for drying the wells and a 1% crystal violet solution was applied on to adherent biofilms and stained it for 15 to 20 minutes then Removed the stain by washing it with water and used ethanol for destaining to release crystal violet finally measured the OD of the solution, at 590 nm using a microplate reader.(10)

5-Genomic DNA was obtained from cultures using the DNA Mini Kit (Qiagen) as per the instructions provided by the manufacturer.

- The cells were collected from a portion overnight culture by centrifugation at 13000 rpm3 minutes. Carefully the part was removed without disturbing the cell pelletand 20µLproteinase added the cell pellet was re-suspended in the lysis buffer provided in the kit.
- The lysate was transferred to a spin column. Then Centrifuge binds DNA to the column matrix while removing debris and proteins.
- Then washing buffers provided in the kit are used for washing to eliminate impurities and salts.
- Finally, the purified DNA was obtained from the column using a low-salt elution buffer. The concentration and purity of the extracted DNA were determined by using a spectrophotometer (Nanodrop).(11)

6-PCR amplification and gel electrophoresis.

Table (1): steps for PCR for *16S rRNA* and *blaIMP* Gene

Step	PCR for *16S rRNA* Gene	PCR for *blaIMP* Gene
Total Volume	25 µL	
Components	▪ 12.5 µL PCR Master Mix ▪ 1 µL Forward Primer (10 µM) ▪ 1 µL Reverse Primer (10 µM) ▪ 2 µL DNA Template ▪ 8.5 µL Nuclease-free Water	
Initial Denaturation	95°C for 5 minutes	
PCR Cycling Conditions (30x)	▪ Denaturation: 95°C for 30 seconds	
	▪ Annealing: 58°C for 30 sec.	▪ Annealing: 57°C for 30 sec.
	▪ Extension: 72°C for 1 minute	
Gel Electrophoresis	▪ PCR products were analyzed on 2% agarose gel	

7-Antimicrobial activity assay of Q.D.

The samples of Q.D suspensions were prepared to make four concentrations (2.5, 2, 1.5, 1 mM) from the stock After that, microbial cultures were prepared in a growth medium (Mueller Hinton). Following that, microbial cultures were inoculated after the suspension of Q.D in four wells onto petri dishes.

Cultures were incubated at 37°C for 24 h measuring inhibition zones around areas treated with Q.D. After incubation to assess effectiveness indicate activity of the four concentrations.

8-Cytotoxic Effect of nanoparticles on HepG2 cancer cell line MTT assay

Using a kit of MTT (Bio-Rad, USA) and micro-titer plates, various concentrations of nano particles (25, 50, 100, 200, and 400µg/mL) were utilized to assess the cytotoxic effects of quantum dots on HepG2 *in vitro* (96-well).(12) described cell line maintenance and method as follows: after the cells in the vessel formed a confluent monolayer and were rinsed with PBS, 3 ml of Trypsin-versine solution was added, and the cells were then incubated at 37 °C for 1 to 2 minutes. Following this, 15 ml of RPMI medium was added, and the cells were further incubated at 37 °C in an incubator with 5% CO_2 until the full concentration of cells was achieved by counting the cells using a hemocytometer. HepG2 cells ($1 \times 10^{-4} \times 10^{6}$ cells mL^{-1}) were grown at 37 °C for 24 hours after being seeded at the final volume (200 microliters). Subsequently, the N.P. concentrations ranging from 25 to 400µg/mL were introduced into each well and allowed to incubate for a full day. After that, 10 microliters of MTT solution were added, and the mixture was further incubated for four hours at 37 °C and 5% CO_2. After extracting the medium, 100 mL of the kit's solubilization solution was added, and the mixture was then further incubated (5 min). To ascertain whether formazan was forming, an ELISA microplate reader operating at 570 nm was employed.

9- Statistical Analysis:

Data were analyzed using GraphPad Prism version 8, and results were presented as mean ± standard deviation (SD). The IC_{50} values for cytotoxicity were determined by nonlinear regression analysis. Significance levels were interpreted based on dose-dependent response trends observed in MTT assays.

RESULTS

1. Biochemical detection of *Pseudomonas aeruginosa*.

The result of biochemical detection of 20 *Pseudomonas aeruginosa* isolates revealed that bacterial isolates were indole negative. On the other hand, the Catalase test showed a positive result. Additionally, the Citrate Test reveals positive results.

2. Antibiotic susceptibility testing

The result of the antibiotic susceptibility test indicated that *Pseudomonas aeruginosa* on a 10-cm Mueller-Hinton agar plate presented in Table (2) produced pyocyanin, which is blue, and pyoverdine, which is green. Together these pigments produce the blue-green color that is seen in the agar around the pseudomonas growth. As shown in Figure (1).

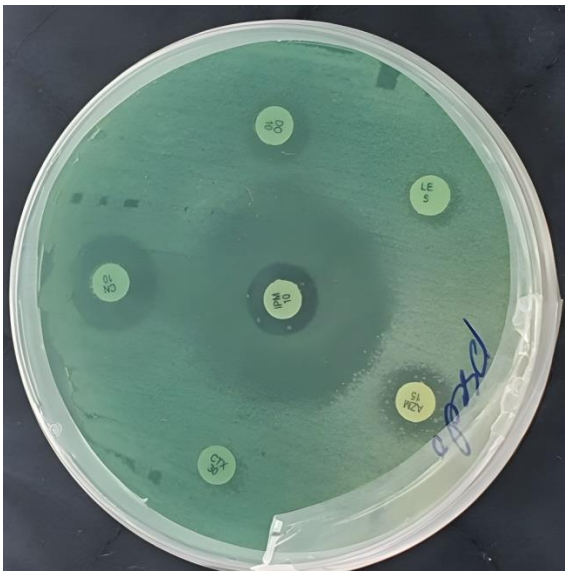

Figure (1): explained antibiotic susceptibility test of *Pseudomonas aeruginosa* and production of pyocyanin.

Table (2): Inhibition zone by different antibiotic against *Pseudomonas aeruginosa*

No.	Types of Antibiotics	Inhibition zone (mm)	X^2	P value	No.	Types of Antibiotics	Inhibition zone (mm)	X^2	P value
1.	Imipenem	10	55.80	<0.0001	4.	Cefotaxime	0	0	0
2.	Doxycycline	6	42.20	<0.0001	5.	Gentamicin	10	55.90	<0.0001
3.	Levofloxacin	2	17.30	0.0002	6.	Aztreonam	8	42.20	<0.0001

3. Biofilm formation.

The absorbance results showed that bacterial isolates under study had grown on the microtiter well's surface and the results were divided into four groups: 10% weak, 30% moderate, 60% strong, and non-biofilm producer. Based on the results given in Table (3).

Table (3): Biofilm intensity of *P. aeruginosa* isolate

ID Biofilm	intensity	OD 630 Limits number of isolates	Percentage of isolates	X^2
None	< 0.0518	0	0	0
Weak	0.0518-0.127	2	10	11.25
Moderate	0.0127-0.230	6	30	88.41
Strong	≥ 0.230	12	60	35.54

4. Molecular detection of *Pseudomonas aeruginosa*

The molecular detection assays were used to analyze the results of both the 16S rRNA gene and the *bla IMP* gene on 6 isolates (2 from ear infections; 2 from urinary tract infections catheter infections; 2 from wound infections). This involved conducting PCR amplification, and gel electrophoresis. During the detection of the 16S rRNA gene primers, positive controls showed bands of 900 bp. The gel electrophoresis of sample lanes revealed bands indicating amplification as shown in (Figures 2 and 3).

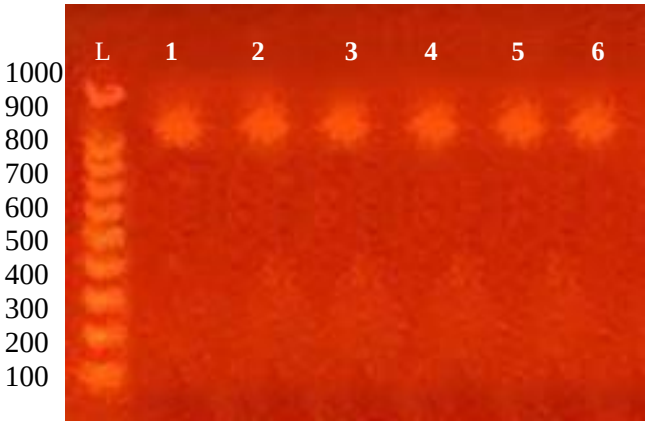

Figure (2): Agarose gel electrophoresis of PCR products after amplification by 16s r RNA gene. marker (100 bp DNA ladder Geneaid), DNA product samples 1,2,3,4,5,6 of different strains of *P. aeruginosa.*

Table (4): The primer sequences for 16sRNA in *P. aeruginosa* strains that are utilized in PCR tests

Primer name	Sequence	Product size
16SrRNA-F	5-GGGGATCTTCGGAACCTCA3	956
16SrRNA-R	5-TCCTTAGAGTGCCCAAACCCG 3	956

Similarly for the IMP gene primers positive controls displayed bands, at the expected size of 700 bp. Gel electrophoresis showed bands in samples indicating successful *bla* $_{IMP}$ gene amplification.

These findings suggest that there are strains of *P. aeruginous* that carry the IMP gene. This information gives us insights into their potential to cause disease and their resistance to antibiotics. As shown in Figure (3).

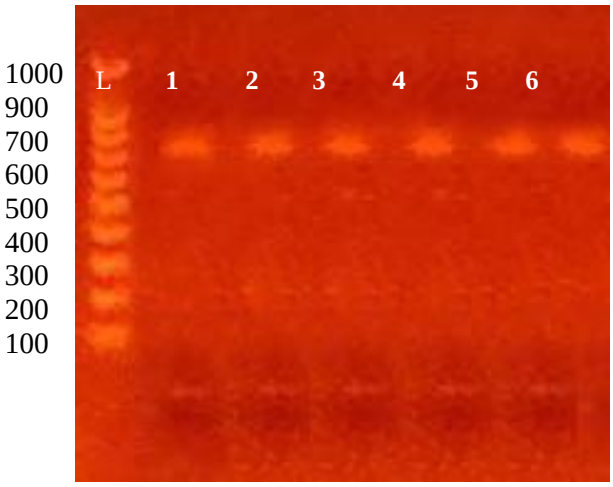

Figure (3):Agarose gel electrophoresis of PCR products after amplification by bla $_{IMP}$ gene. marker (100 bp DNA ladder Geneaid), DNA product samples 1,2,3,4,5,6 of different strains of *P. aeruginosa.*

Table (5): The primer sequences forvirulence genes in *P. aeruginosa* strains that are utilized in PCR tests

Primer name	Sequence	Product size
bla IMP-F	5-TGAGCAAGTTATCTGTATTC-3	740
bla IMP-R	5-TTAGTTGCTTGGTTTTGATG-3	

5. Antibacterial activity of quantum dots nanoparticle

Four concentrations of QDs (100, 75, 50, and 25 µg/ml) were applied to culture media after it was determined that CQDs may demonstrate higher microbicide potency against *pseudomonas aeruginosa*. The result showed a 100% inhibition zone, (13) as shown in figure (4) and table (6).

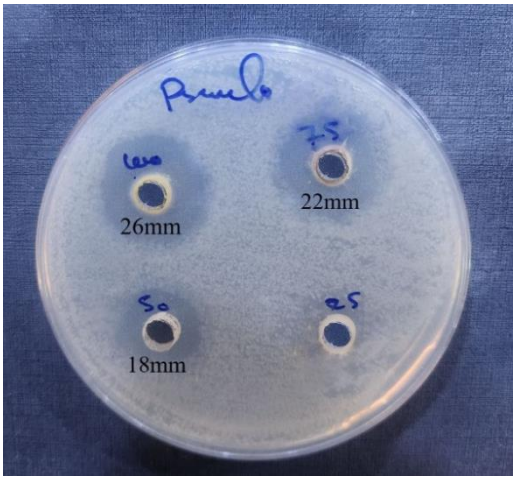

Figure (4): The effect of nanoparticles on *Pseudomonas aeruginosa* agar plate.

Table (6): Inhibition zone of Q.D nanoparticles against *Pseudomonas aeruginosa*

Conc. (mg/mL)	100	75	50	25
Inhibition zone (mm)	26	22	18	---

6. Utilizing the MTT assay, the nanoparticle's cytotoxic effects

The result of the MTT assay of Q.D on HepG2 released that cell viability decreased concentration of Q.D increased and maximum inhibition for cell viability at 400µg/ml (49.03±3.1) in compression to 25 µg/ml which showed cell viability percentage at (83.26±0.69). Q.D possessed the maximum cytotoxic activity within IC50 of 305.7 obtained from the effect of Q. D obtained from the effect of Q.D on normal cell line (Hdfn) with cell viability ranging from (96.3±1.4 - 67.43±2.15) for 25 to 400µg/ml respectively (Table7, Figure 5).

Table (7): Cytotoxicity effect of Q.D on liver cancer (HepG2) and normal cell line (HdFn).

Q. D. s Conc. (µg/ml)	Viable cells count %		P value
	HepG2 (Mean± S.D)	HdFn (Mean ± S.D.(%)	
400	49.03±1.10	67.43±2.15	<0.01
200	55.20±1.77	78.95±1.37	<0.01
100	63.42±1.48	84.33±1.50	<0.01
50	75.61±1.70	93.20±1.20	<0.01
25	83.26±0.69	96.3±1.40	<0.01

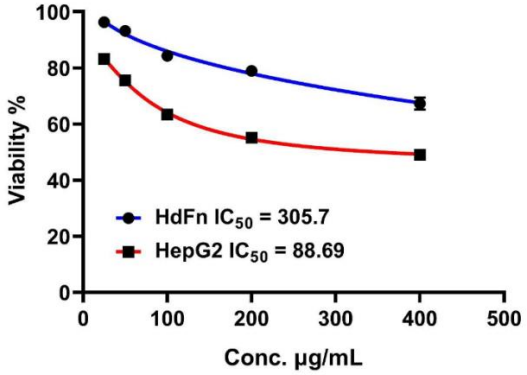

Figure (5): Cytotoxicity effect of nanoparticles (Q. D) on HepG2 and HdFn cells using MMT assay.

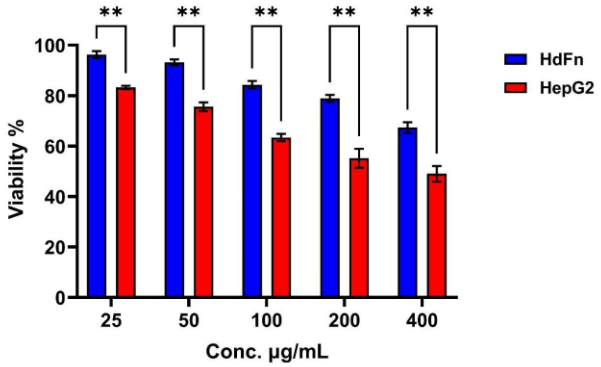

Figure (6): Cytotoxic effect of nanoparticles (Q. D) on HepG2 and HdFn cells using MMT assay.

DISCUSSION

Pseudomonas aruginosa is known for its adaptability and capacity to infect people, animals, and plants. Inaccurate identification may affect the antibiotic susceptibility testing, and administration of effective antipseudomonal antibiotics(14,15), In the current study, we identified the antimicrobial agents of Imipenem, Ciprofloxacin, Ceftazidime, Amikacin, Gentamicin, and Aztreonam using Mueller Hinton medium and growth conditions that agree with (16). This antimicrobial agent (medication) is usually used as an adjutant in the treatment of some chronic lung infections due to its anti-inflammatory action as a result of its capacity to decrease the immune system response(17).

In this study, the results indicate that *Pseudomonas aeruginosas* biofilm formation offers defense against a range of strengths (high). A biofilm is a structure that serves as a shield against different environmental pressures such, as antibiotics and the body's immune response. These substances create a framework around the cells offering them stability and shielding them from challenges like antibiotics and immune reactions. The EPS framework also aids in the attachment of cells to surfaces. Enables communication between cells. To make specific detection the use of techniques allows the identification of *Pseudomonas aeruginosa* infections, which helps in starting the right treatment promptly (18, 19). In this research the detecting *Pseudomonas aeruginosa* rapidly and accurately through methods assists healthcare providers in making informed choices regarding patient care, such, as selecting the appropriate antibiotics and implementing infection prevention strategies to avoid spreading to other patients. Additionally detecting the bla_{IMP} gene in *Pseudomonas aeruginosa* using methods is a tool, in fighting antimicrobial resistance and enhancing patient outcomes by guiding treatment decisions based on reliable information(20).

Using MTT tests, Hbg2 cells and noncancerous human cells (HdFn) were examined. Interestingly, our Q-dots showed the anticancer activity by the growth inhibition of *cancer* cells with a half-maximum inhibitory concentration (IC50) between, Q-dots were nontoxic to HbG2 cells up to the concentration of 500µg/mL with the viability of over cells treated with Q-dots.

There are numerous biological and environmental applications for quantum dots. The electrostatic intercalative binding to DNA has been demonstrated by C-dots. It demonstrated outstanding antibacterial activity against *Pseudomonas aeruginosa* since quantum dots can exhibit effects by producing reactive oxygen species (ROS) in response to light (21). These quantum dots can produce toxic ROS for bacteria, such as superoxide and singlet oxygen when they are light. The cell dies because of damage to the cell membrane and other essential cellular components brought on by this oxidative stress. As well as anticancer actions owing to the many benefits of the environmentally friendly and green method, our Q-dots are anticipated to pave new paths in environmental chemical sensing as well as antibacterial and cancer nanomedicine applications(22).

CONCLUSION

Pseudomonas aeruginosa isolates were sensitive to Imipenem, Doxycycline, Levofloxacin, Cefotaxime, Gentamicin, Aztreonam at varying inhibition zones and quantum dots nanoparticles possessed the activity to inhibit the bacteria 100%.in addition to that quantum dots possessed cytotoxicity against cancer cell lines.

Acknowledgment

Characterization studies explore the composition of these isolates pinpointing the genetic components linked to antibiotic resistance. Molecular detection methods, like polymerase chain reaction (PCR) or genome sequencing, facilitate the detection of the *blaIMP* gene, in the bacterial genome.

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التوصيف والكشف الجزيئي لجين *blaIMP* من العزلات السريرية لبكتيريا *Pseudomonas aeruginosa* وانعكاساتها على مقاومة المضادات الحيوية وتكوين الأغشية الحيوية

هديل محمد خلف

التقنيات الحيوية الجزيئية والطبية، التقنيات الاحيائية، النهرين ، العراق

الخلاصة:

خلفية عن الموضوع: يتركز هذا البحث على تحديد جين *blaIMP* في عينات من بكتيريا *Pseudomonas aeruginosa* مما يوفر نظرة ثاقبة حول تأثيره على المقاومة وتكوين الأغشية الحيوية من خلال استخدام طرق وتعزيز الثبات وتعقيد العلاج للعدوى التي تسببها هذه الكائنات الحية الدقيقة. **الهدف من الدراسة:** الكشف الجزيئي لجين *blaIMP* ، في عينات *Pseudomonas aeruginosa* التي تم الحصول عليها من المرضى وتقييم علاقتها بالمقاومة ضد المضادات الحيوية وتطور الأغشية الحيوية. فهم أهمية جين *blaIMP* من حيث المقاومة وتأثيره على الثبات المرتبط بالأغشية الحيوية. التحقق من الأساليب التي يمكن استخدامها لمعالجة المقاومة التي يسببها *blaIMP* وتكوين الأغشية الحيوية في حالة عدوى *Pseudomonas aeruginosa*. **طرق ومواد العمل:** تم جمع عشرين عزلة من *Pseudomonas aeruginosa* مستشفى مدينة الطب التعليمي لاختبار تطور الأغشية الحيوية وتحديد جينات الفوعة باستخدام الوسائل الجزيئية واكتشاف طرق لمقاومتها و تقليل مقاومة المضادات الحيوية. **النتائج:** أظهرت نتائج الكشف الكيموحيوي لعشرين عزلة من عزلات *Pseudomonas aeruginosa* أن العزلة البكتيرية كانت سلبية للإندول بينما كانت العزلات السيترات والكاتليز موجبة وكانت هذه العزلات منتجة لصبغة البايوسيانين وحساسة لكل من الإيميبينيم والدوكسيسايكلين والليفوفلوكساسين والسيفوتاكسيم والجنتاميسين والأزتريونام وبنطاق تثبيط قدره 10. 8،10،0،2،6 على التوالي. أشارت نتائج تكوين الأغشية الحيوية لـ 20 عزلة مختارة إلى أن 10% ضعيفة، 30% متوسطة، 60% قوية، وغير منتجة للأغشية الحيوية. أيضًا، كشف تضخيم PCR المستخدم للكشف عن العزلات البكتيرية لل *Pseudomonas aeruginosa* أنه بواسطة جين 16 S rRNA وجين*bla IMP* ، حيث ظهرت حزم في التضخيم. وأظهرت نتائج النشاط المضاد للبكتيريا للجسيمات النانوية *Pseudomonas aeruginosa* أنه عند أربعة تراكيز تم اختبارها (100،75،100،75،100). أظهرت جميع المعزولات المختبرة منطقة التثبيط. وأخيرًا، كشفت نتائج فحوصات السمية الخلوية للجسيمات النانوية باستخدام اختبار MTT على خط سرطان الكبد HepG2 أن حيوية الخلية تعتمد على التركيز، وأنها انخفضت مع زيادة التركيزات إلى 75.61±1.70 إلى 49.03±3.1. تم الحصول على 50 إلى 400 ميكروغرام/مل على التوالي ضمن قيمة IC50 البالغة 88.69 بينما قيمة IC50 البالغة 305.7 تم الحصول عليها من تأثير الجسيمات النانوية على خط الخلية الطبيعي .(HdFn) **الاستنتاجات:** ان جميع العزلات كانت حساسة للإيميبينيم، السيبروفلوكساسين، السيفتازيديم، أميكاسين، الجنتاميسين، والأزتريونام عند تثبيط متفاوت. تمتلك المناطق والجسيمات النانوية نشاطًا مثبطًا للبكتيريا بنسبة 100٪. بالإضافة إلى أنها تمتلك سمية خلوية ضد خط الخلايا السرطانية.

الكلمات المفتاحية: *Pseudomonas aeruginosa* ، النقاط الكمومية ، سرطان الكبد ، اختبار MMT .