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Molecular Detection of Aminoglycosides Modifying Enzymes and It's Relationship to Drug Resistance in *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen that causes a variety of infections throughout the world, including bacteremia in immunocompromised patients, wound and soft tissue infections, hospital-acquired pneumonia, respiratory infections, and urinary tract infections (UTI). It is also the primary cause of life-threatening infections in burn patients. A total of 120 specimens that collected of Al-Anbar Province hospitals, they obtained from a wound (65.85%), burns (52.63), UTI (33.33%), and sputum (48.27%). Out these specimens, 65 strains identified as *Pseudo.aeruginosa* depending on microscopical and cultural properties, automatedVITEK-2 system, and the molecular identification based on *16SrRNA* gene, which is the essential in all *Pseudo.aeruginosa*. The antibiotics susceptibility test was done by using disc diffusion method of aminoglycoside antibiotics.

The results were indicated, that the highest resistance to Gentamycin(10µg) was (81.53%), Amikacin(30µg) (73.84%), then Tobramycin(10µg) (66.15%), and Netilmicin(30µg) (33.84%). Molecular analysis of aminoglycosides modifying enzymes genes showed that the percentage of the *AAC (6 ')-Ib* gene was highest 16/20 (85%), then 8/20 (40%), 2/20 (10%) for *ANT (3 ")-Ia , APH(3 ')-IIb*, respectively.
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Introduction:

Pseudomonas aeruginosa is an opportunistic pathogen that causes a variety of infections throughout the world, including bacteremia in immunocompromised patients, wound and soft tissue infections, hospital-acquired pneumonia, respiratory infections, and urinary tract infections. It is also the primary cause of life-threatening infections in burn patients[1,2]. It is well-recognized that *Pseudo. aeruginosa* can be challenging to treat with antibiotics and antiseptics. As a result of the widespread use of antibiotics to treat illnesses brought on by these bacteria, antibiotic-resistant strains have emerged and have grown more resistant over time, making it one of the most prevalent issues, especially for patients who are still hospitalized (Saxena *et al*., 2014). The related resistance of *Pseudo. aeruginosa* is typically divided into acquired or mutational resistance and intrinsic/innate resistance[3].

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The low permeability of the outer membrane, the production of inducible enzymes, and the presence of several groups of efflux pumps are all thought to contribute to *Pseudo. aeruginosa* intrinsic resistance[4]. In addition, *it* can capture and ingest gene clusters that confer antibiotic resistance and increase virulence. Due to the acquisition of mobile elements like Integrins and the accompanying antibiotic-resistance gene cassettes, drug-resistant *Pseudo. aeruginosa* strains have increased dramatically.

Antimicrobial aminoglycosides are lethal to bacteria because of their capacity to attach of the (30S) ribosome during the translation process of the mRNA, causing misreading and hence preventing protein synthesis .The most often used aminoglycoside antibiotics for the treatment and control of *Pseudo.aeruginosa* infections are tobramycin, amikacin, and gentamicin. Many processes may contribute to the development of aminoglycoside resistance, including increased activity of the MexXY-OprM efflux pump, acquired mutations, and enzymatic modification and inactivation of aminoglycosides[5] .

The most common genetically coded modifications that *Pseudo. aeruginosa* strains have against aminoglycosides are acetyltransferase (AAC), nucleotide transferase (ANT), and phosphotransferase (APH) . Increased antibiotic resistance among clinical *Pseudo. aeruginosa* strains are main causes of therapeutic failure. Therefore, for effective management of this problem, it is crucial to identify the underlying mechanisms of resistance.

Methods:

Collection and Isolation of Samples :

Clinical samples were taken from wounds, burns, urine, and sputum. 65 strains of *Pseudo. aeruginosa* were obtained from one hundred twenty samples and were collected from affected patients from Al-Anbar Province Hospitals, during the period from September 2022 to December 2022. Cultural characteristics, microscopical, biochemical tests, and automated (VITEK-2 system), are used to identify *P. aeruginosa*.

Antimicrobial Susceptibility Test (AST):

The disk diffusion method, also called the Kirby-Bauer method is a standardized system for testing the efficacy of antimicrobials against micro-organisms. Isolated pure colonies (2-4) were inoculated in brain-heart broth (2ml) to obtain a bacterial suspension, which was compared with a McFarland turbidity tube $1x10^8$CFU/ml, Muller Hinton agar plates were inoculated using cotton swabs from that prepared suspension and incubated under standard condition. Anti-microbial discs were positioned upon the medium's surface, then incubated 37°C of 24 hours. For interpreting the results, the Clinical and Laboratory Institute's standards were followed (Clinical and Laboratory Institute Standards 2022).

Molecular Identification:

Molecular detection of (*AAC (6 ')-Ib , ANT(3 '')-Ia , APH(3 ')-IIb*) genes by using PCR technique .

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

Genes	Primer sequence (5′-3′)	Size (bp)	Reference
16SrRNA	F-AGAGTTTGATCCTGGCTCAG	1500 bp	[6]
	R-CTACGGCTACCTTGTTACGA		
AAC(6 ')-Ib	F- GAC CAA CAG CAA CGA TTC	375b p	[7]
	R- AAC AGC AAC TCA ACC AGA		
ANT(3 '')-Ia	F- TGT AGA AGT CAC CAT TGT TG	15 2b p	[7]
	R- TCA GCA AGA TAG CCA GAT		
APH(3 ')-IIb	F- TTC GTC AAG CAG GAA GTC	662bp	[7]
	R- TAG AAG AAC TCG TCC AAT AGC		

***F: Forward sequences, R: Reverse sequences**.

Preparation of PCR mixture:

The PCR reaction mixture (25µl) was composed of green master mix (Promega) (12.5µl), Forward primer solution(1.0µl), Reverse primer solution(1.0µl), deionized water(8.5µl), and template DNA (2.0µl)

Table (2): Programs of a PCR reaction.

Genes	Size (bp)	Steps	PCR condition ^{0}C(min)
AAC(6 ')-Ib	375bp	Initial denaturation	94 (4) 1x
		Denaturation Annealing Extension	94 (1) 57 45 sec 35x 72 (1)
		Final extension	72 (7) 1x
ANT(3 '')-Ia	152bp	Initial denaturation	94 (4) 1x
		Denaturation Annealing Extension	94 (1) 51 35 sec 35x 72 45 sec
		Final extension	72 (7) 1x
APH(3 ')-IIb	662bp	Initial denaturation	94 (4) 1x
		Denaturation Annealing Extension	94 (1) 54 30 sec 35x 72 45 sec
		Final extension	72 (7) 1x
16SrRNA	1500bp	Initial denaturation	94 (4) 1x
		Denaturation Annealing Extension	94 (1) 58 25 sec 35x 72 30 sec
		Final extension	72 (7) 1x

Results and discussion:

Isolation of *Pseudo .aeruginosa* :

Sixty-five strains were confirmed as *Pseudo.aeruginosa* from a total of one hundred twenty specimens (burn, wound, sputum, and UTI). The strains (65.85%) were found in wound samples. whereas that from burns were (52.63) strains, these results agreed with [8] that found *Pseudo.aeruginosa* is one of the more bacterial that cause wounds and burns infection in hospital, at a rate of (69%)of wounds and(53%)of burns. While the result is not consistent with [9] who found that *Pseudo.aeruginosa* only in (10%) of the wound samples. Compared to other patients, burn patients and wounds have a greater probability to get infections because of their damaged skin barrier, weakened immune system, extended hospital stay, and invasive therapeutic and diagnostic treatments . In addition, a large percentage of burn infections may be caused by *Pseudo. aeruginosa* contamination of hospital air, tools, and workers' hands, due to its ability to grow on moist burn wound areas of skin and remain alive in hospital environments.

Other infection percentages represent (33.33%) of UTI, (48.27%) from sputum infection of total clinical strains, close to [10] the percentage of UTI that he recorded was (41.37%), but this percentage does not correspond to [11] which indicated the percentage was (6.3%) of UTI. [12] showed that the percentage of these bacteria from sputum was (29.3%). *Pseudo. aeruginosa* may be involved in UTIs as a nosocomial infection as a result of the placement and removal of indwelling urinary catheters[13] .

Antimicrobial susceptibility test:

A large percentage of *Pseudo. aeruginosa* strains showed high levels of resistance was (81.53%), (73.84%) for Gentamycin, and Amikacin respectively. this percentage is consistent with (14) who showed (87%) resistance to gentamicin, and (15) that found the *Pseudo. aeruginosa* isolates in Kurdistan of Iraq gentamicin resistant rate (88%). But (16) found that *P. aeruginosa* has less resistance to Gentamicin (50%) than our study. Our study indicates the Gentamycin was slowly effective against *P.aeuroginosa*. Gentamicin is a easily and cheap available drug that used in general and hospital practice in clinically suspected Gram-negative infections. In addition, Resistance to Amikacin was (73.84%) which agreed with (17)and (18) that showed that the percentage of amikacin resistance was 79% and 82% respectively.

The current study shows a percentage of Tobramycin (66.15%) and Netilmicin (33.84%). This result agrees with what was reached by the researcher [19]which determined the resistance to Tobramycin (65.43%), while [20] reported a rate of Tobramycin (78.8%).[21] , found that(20 %) of isolates were resistant to Netilmicin. The bacterium *Pseudo. aeruginosa* has shown resistance to this group, which is related to its secretion enzymes, such as coenzyme N-acetyltransferase and phosphotransferase. That's recognized as many types of aminoglycoside modifying enzymes , whose encoded genes may be portable chromosomally or plasmidally encoded, in addition to another resistance mechanism [22].

Molecular analysis:

Pseudomonas aeruginosa strains was identified using *16SrRNA* gene, this gene is plays an important role in diagnosis, in this study, all isolates exhibit the *16S rRNA* gene, Figure (1).

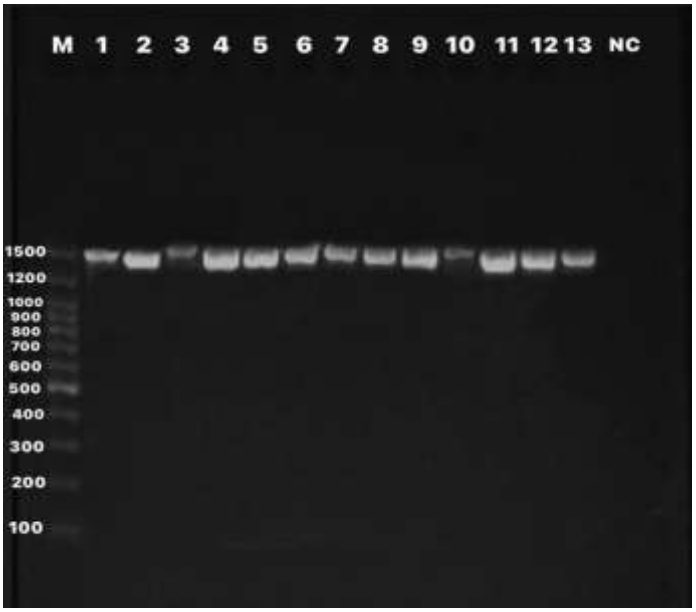

Figure (1): Uni-plex PCR amplification fragments for the detection of *16SrRNA* gene. M:100-bpDNA ladder ; NC: negative Control. Amplicons were electrophoresed on agarose gel

(1.5%) at 70 V/cm for 1.5 h, stained with Ethidium bromide, and visualized using a UV transilluminator documentation system.

A molecular study of twenty strains show that percentage of the *AAC(6 ′)-Ib* gene was highest 16/20 (85%) at size product (375bp), Figures (2). The percentage of *AAC(6')-Ib* in this study was a high compared to another study in Egypt, where those gene was present at a low rate 42.2% [23]. In Iran, it was (71.2%)[24] , and [25]in Saudi illustrated that *AAC(6')-Ib* gene was detected (71.8%) of *Pseudo .aeruginosa* strains. Reason the *AAC(6')-Ib* is widely distributed among bacteria is that the gene is frequently found on mobile elements, which facilitates its rapid transfer's[19] .

After that, 8/20 (40%),2/20 (10%) for *ANT (3")-Ia ,APH(3 ′)-IIb*, respectively. The product size of the *ANT(3")-Ia* (152bp) and the gene *APH (3 ′)-IIb* (662bp) confirmed by using electrophoresis, as displayed in Figures [3,4]. In Tehran, the ratio of *ANT(3 ″)-Ia* was different from we obtained in our study; it was 87.5[26]. Other study, the presence of *ANT(3")-Ia* was 18.6% [7]. The result of *APH(3')-IIb* in this study differed from [26] which reported the high prevalence of *APH(3')-IIb*(60%). In another study, the presence of *APH(3')-IIb* was 34%[7].

The variations in prescription patterns for aminoglycosides, the choice of a bacterial population, regional variations in prevalence of aminoglycoside resistance genes may all contribute to variations in the distribution of aminoglycoside modifying enzymes. Other processes that might result in resistance to aminoglycosides include variations in permeability of outer membrane, mutation-induced changes to the 30S ribosomal subunit, and active efflux systems . As a crucial regulatory process in prokaryotes, phosphorylation of proteins involves the removal or addition of phosphoryl groups. Phosphorylation can affect protein function through controlling protein conformation, protein-protein interactions ,and subcellular localization [27]. In addition, *ANT* enzymes enable aminoglycoside inactivation by catalyzed the transfer of adenosine monophosphate group from donor substrate adenosine triphosphate to the hydroxyl group in aminoglycoside molecule's [28].

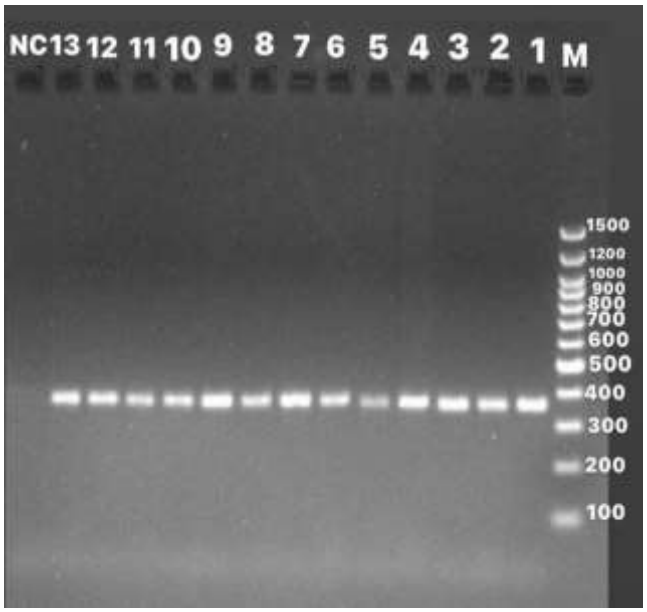

Figure (2): Uni-plex PCR amplification fragments for the detection of aac(6 ′)-Ib gene (375bp). M:100-bp DNA ladder. Amplicons were electrophoresed on agarose gel (1.5%) at 70 V/cm for 1.5 h, stained with Ethidium bromide, and visualized using a UV transilluminator documentation system.

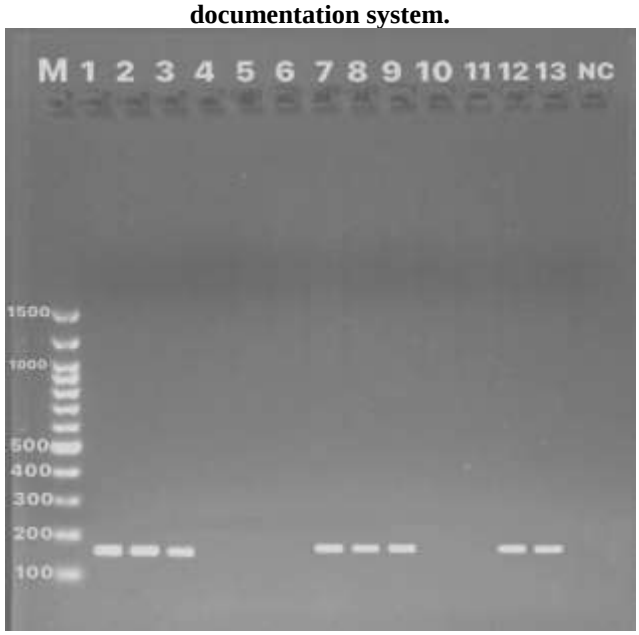

Figure (3): Uni-plex PCR amplification fragments for the detection of ant(3 ″)-Ia gene (152bp). M:100-bp DNA ladder. Amplicons were electrophoresed on agarose gel (1.5%) at 70 V/cm for 1.5 h, stained with Ethidium bromide, and visualized using a UV transilluminator documentation system.

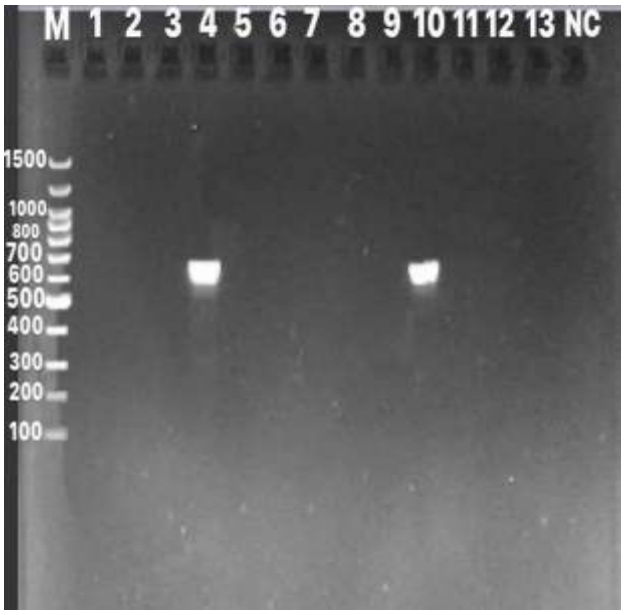

Figure (4): Uni-plex PCR amplification fragments for the detection of *aph (3 ′)-IIb* gene (662bp). M 100-bp DNA ladder. Amplicons electrophoresed on the agarose gel (1.5%) at 70 V/cm for 1.5 h, stained with Ethidium bromide, and visualized using a UV transilluminator documentation system.

Conclusion:

In the current study, the Prevalence of *Pseudo.aeruginosa* resistance to aminoglycosides in hospitals and the acquisition of resistance genes encoding for them is a serious problem that threatens communities. Therefore, it is necessary to find solutions to reduce this resistance and work to find therapeutic alternatives to these antibiotics to reduce this problem.

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الكشف الجزيئي عن الإنزيمات المحورة للأمينوغليكوسيدات وعلاقتها بمقاومة الأدوية في الزوائف الزنجارية

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الخلاصة:

بكتريا الزوائف الزنجارية تعد أحد مسببات الأمراض الانتهازية التي تسبب مجموعة متنوعة من العدوى في جميع أنحاء العالم، بما في ذلك تجرثم الدم في المرضى الذين يعانون من نقص المناعة، والتهابات الجروح والأنسجة الرخوة، والالتهاب الرئوي المكتسب من المستشفيات، والتهابات الجهاز التنفسي، والتهابات المسالك البولية. كما أنها المسبب الرئيس للعدوى التي تهدد الحياة في مرضى الحروق. جمعت 120 عينة من مستشفيات محافظة الانبار ، تم الحصول عليها من الجروح (65.85%) ، الحروق (52.63%) ، التهاب المسالك البولية (33.33%) ،البلغم (48.27%)، تم التعرف على 65 سلالة على أنها *Pseudo.aeruginosa* اعتمادًا على الخصائص الزرعية والميكروسكوبية ، ونظام VITEK-2، وتم التشخيص الجزيئي بناءً على جين 16SrRNA ، وهو جين أساسي معبر عنه في جميع السلالات. تم إجراء اختبار الحساسية للمضادات الحياتية باستخدام طريقة الانتشار القرصي أمينوغليكوزيد. أظهرت النتائج ان أعلى مقاومة للجنتاميسين (81.53%)، أميكاسين (73.84%)، توبرامايسين (66.15%) ، نيتلمايسين (53.84). أظهر التحليل الجزيئي لجينات الإنزيمات المعدلة للأمينوغليكوسيدات أن النسبة المئوية لجين AAC (6 ′) -Ib كانت الأعلى 20/16 (85٪) ، ثم 20/8 (40٪) ، 20/2 (10٪) لـ ANT (3 ″) -Ia، ANT -IIb (3 ′) APH ، على التوالي.