Dissemination of Aminoglycosides Resistance in *Pseudomonas aeruginosa* Isolates in Al-Nasseryia Hospitals

إنتشار مقاومة المضادات الأمينو كلايكوسيديه لعزلات *Pseudomonas aeruginosa* في مستشفيات مدينة الناصرية

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الخلاصة

خلفيه البحث: الزوائف الزنجاريه من أكثر أنواع الجراثيم المرضية الانتهازية والمتسببة في انتشار العدوى ما بين المستشفيات. الأمينوكلايكوسايد هو من المضادات الحيوية الكيميائية المهمة المستخدمة ضد هذا النوع من الجراثيم . إن عمليه تثبيط ميكانيكيه عمل هذا المضاد يكون أما عن طريق تغيير الأنزيمات ذات العلاقة بهذا المضاد أو أفراز أنزيم 16SrRNA methylase.

الهدف: أن الهدف من هذه الدراسة هو تحديد انتشار مقاومة مضادات الأمينوكلايكوسايدز في عزلات الزوائف الزنجاريه المعزولة من العينات السريرية للمرضى الراقدين والوافدين في مستشفيات مدينه الناصريه.

المنهجية: تمّ جمع 355 عينة سريرية من ثلاث مستشفيات رئيسه في الناصرية وقُسمت العينات بحسب مصادر جمعها إلى ثلاث مجموعات (160 مسحة أذن و 105 مسحة حروق و 90 عينة إدرار)، حيث أظهرت نتائج الفحوصات الزرعية والكيموحيوية عائديه 65 عزله لبكتيريا الزائفة الزنجاريه*Pseudomonas aeruginosa* ، وتم تأكيد تشخيصها بواسطةAPI-. 20E أختبرت حساسية العزلات تجاه 18 نوعاً من المضادات الحيوية بطريقة إنتشار القرص لـ كيربي- باور. تم الكشف عن تواجد جينات كل من AACs, APHs, ANTs ,16rRNAmethylase, للعزلات المقاومة لنوع واحد على الأقل من الأمينوكلايكوسايد باستعمال تقنية سلسلة تفاعل انزيم البلمرة PCR.

النتائج: أظهرت النتائج عائديه 65 عزله لبكتيريا الزائفة الزنجاريه من مجموع 355 عزله ، كما بينت نتائج اختبار الحساسية لتلك البكتيريا أن هناك 29 عزلة (44.6%)مقاومه على الأقل لنوع واحد من مضادات الأمينوكلايكوسيديه المستعملة في هذه الدراسه والمتمثلة بمضادات الأميكاسين و الجنتامايسين و التوبرامايسين و النتلمايسين حيث كانت أعلى نسبه للجنتامايسن (44.6%) و أقل نسبه للأميكاسين (18.5%)0 كما أظهرت النتائج أن هناك أربعة من الجينات المشفرة للأنزيمات الناقلة لمجموعة الاستيل ACC موجوده في (21) عزله من العزلات المقاومه للأمينوكلايكوسايدز حيث كان الجين *aac(6')-Ib* هو الأكثر تواجدا بنسبة (69%) يليه *aac(6')-I* بنسبه (24.1%) أما كل من الجينين *aac(3)-II* و*aac(3)-I* فقد ظهرا بنسبة على التوالي (10.3%) على التوالي .وأوضحت الدراسه الى وجود تكرارا مميز لجينين كانا قد ظهرا في العزلةالواحدة حيث كانت نسبة *aac(6')-Ib+aac(6')-I* و *aac(3)-II + aac(6')-Ib* (12.7%) و(6.9%) على التوالي .أما جينات 16SrRNA methylase*(armA, rmtA, rmtD and npmA)* و*aph (3')-VI* and *ant (4')-IIb* genes فلم يتم الكشف عنها في جميع العينات .

الاستنتاج : هناك زيادة ملحوظه في مقاومة عزلات الزوائف الزنجاريه لمضادات الأمينوكلايكوسايدز وقد كان المورث *aac(6')-Ib* هو الغالب عن بقية المورثات.

التوصيات : وضع خطة عمل شامله لتحديد وتعين نوع العلاج من المضادات الحياتية وخاصة مجموعة الامينوكلايكوسايدز لمنع اتساع المقاومة لها من قبل بكتيريا الزوائف الزنجاريه.

Abstract

Background: *Pseudomonas aeruginosa* is one of the primary opportunistic pathogens responsible for nosocomial infections. Aminoglycosides are an important component of antipseudomonal chemotherapy. The inactivation of drugs by modifying enzymes and 16S rRNA methylase are the most common mechanisms of aminoglycoside resistance.

Aim of the study: Investigate the occurrence of aminoglycoside resistance and the incidence of the 16S rRNA methylase and resistance-modifying enzyme genes in *P. aeruginosa* isolated from several hospitals in Al-Nasseryia province.

Methods: Atotal of 355 clinical isolates were collected from three main hospitals in Al-Nasseryia city. The isolates were classified according the source of collection to three groups (160 ear swab, 105 burn swab and 90 urine samples). The results of culture and biochemical tests showed that 65 isolate was *Pseudomonas aeruginosa*, which then confirmed by API-20E. Antibiotic susceptibility test (by using the Kirby-pour technique) was performed to all these isolates by using 18 type of antibiotics. The isolates that resistance to at least one type of aminoglycosides were screened for the presence of modifying enzyme genes (AACs, APHs, ANTs)and16S rRNA methylase genes by polymerase chain reaction.

Results:65 (18.3%) isolates of *P. aeruginosa* were identified.The results of antibiotic susceptibility test showed that 29 isolate (44.6%) resistance to at least one type of aminoglycosides which represented by gentamicin the highest (44.6%) and amikacin (18.5%) the lowest. Four genes of AAC were found in 21 (72.4%) aminoglycosides resistant isolates, *aac(6')-Ib* (69%), *aac(6')-I* (24.1%), *aac(3)-II* and *aac(3)-I* (10.3% each). The most repeated combinations, *aac(6')-Ib+aac(6')-I* and *aac(6')-Ib+aac(3)-II*, were detected in 12.7% and 6.9% of isolates, respectively. Negative results for the presence *16rRNA* methylase (*armA*, *rmtA*, *rmtD*, *and npmA*), *aph (3')-VI* and *ant (4')-IIb* genes.

Conclusions: The aminoglycosides resistance rates in *P. aeruginosa* were roughly high in Al-Nasseryia, and most of the resistance isolates harbored *aac (6')-Ib* gene.
Recommendations: In order to overcome the worrisome development of increased resistance to antibiotics in general and aminoglycoside in particular, continued national surveillance programs are crucial.
Keywords:*Pseudomonas aeruginosa*; Antibiotic; Resistance; Aminoglycosides Modifying Enzymes (AMEs)**.**

INTRODUCTION

Pseudomonas aeruginosa is one of the most common Gram-negative pathogens associated with nosocomial infections [(1)]. *P. aeruginosa* can acquire resistance to various antimicrobial agents, such as aminoglycosides, β-lactams and fluoroquinolones. Aminoglycosides are an important component of antipseudomonal chemotherapy [(2, 3)]**.**

Resistance to aminoglycosides occurs via enzymatic modification, impermeability, the activity of efflux pumps (MexXY-OprM)[(4)], the PhoP-PhoQ system[(5)], ndvB dependent biofilm formation and the activity of 16S rRNA methylase [(6)]. Among these mechanisms, the inactivation of drugs by plasmid-encoded modifying enzymes is the most common. These modifying enzymes include aminoglycoside phosphoryl transferase (*aph*), aminoglycoside acetyltransferase (*aac*), and aminoglycoside nucleotidyl transferase (*ant*) [(7, 8)]. More recently, 16S rRNA methylase genes were identified in *P. aeruginosa*[(9)]. There are few documented data available in Iraq on the resistance of *P. aeruginosa* isolates [(10, 11)]. Moreover, there is no study addressing the pattern of antibiotic resistance of *P. aeruginosa* in Al-Nasseryia province. Thus, the aim of the present study was to investigate the occurrence of antipseudomonal as well as aminoglycoside resistance and the incidence of the 16S rRNA methylase and resistance-modifying enzyme genes in *P. aeruginosa* isolated from several hospitals in Al-Nasseryia province.

METHODOLOGY:

During the period from November 2012 to April 2013, a total of 355 non-duplicate clinical samples were collected from patients visited/or admitted to three main hospitals in the Al-Nasseryia city. The study population was 54.9% male and 45.1% female. The specimens were isolated from ear swab (45.1), wound (29.6%) and urine (25.4%). Isolate confirmations were conducted using conventional biochemical tests and confirmed by the API 20E system, and then the isolates were stored at –76°C in glycerol skim milk broth.

Antimicrobial susceptibility tests were performed by using the disk-diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines[(12)] for four aminoglycosides [amikacin (30 μg), gentamicin (10 μg), tobramycin (10 μg) and netilmicin (30 μg)]. All drugs were obtained from (Bioanalyse company-Turkey). *E. coli* ATCC 25922 served as a control for the disk diffusion test.

Polymerase chain reaction (PCR) was used to screen for the presence of the aminoglycosides modifying enzyme genes:*aac(3)-I, aac(3)-II, aac(6')-I, aac(6')Ib, Aph(3')-IV, Ant(4')-IIb* and16rRNA methylase genes (*armA, rmtA, rmtD and npmA*). The total template DNA for the PCR amplification was extracted from the supernatant of a mixture of *P. aeruginosa* cells produced by salting out method [(13)]. PCR amplification was performed using 5 μl of the template DNA, 2 μl of each primer, 10 μl master mix, and 1 μl of Taq DNA polymerase in a total volume of 20 μl. A thermocycler (Mastercycler gradient; Eppendorf, Hamburg, Germany) was programmed with the appropriate conditions[(13)]. Then, 5μl of each PCR product was analyzed by electrophoresis on 1.5% (w/v) TAE agarose gel containing 0.1 μl/mL ethidium bromide [(14)]. The amplicon were then visualized on a UV transilluminator and photographed (BioDoc-Analyse; Biometra, Goettingen, Germany).

STATISTICAL ANALYSIS:

All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) software. P-values of <0.05 were considered significant. L.S.D. values were compared with values of means difference.

RESULTS:

A total of 355 clinical samples have been collected, the distribution of clinical samples was explained in table 1. 160 (45.1%) were collected from ear while 105(29.6%) and 90 (54.9) samples were collected from burn and urine respectively. According to the gender 160 (45.1) samples were collected from female while 195 (54.9%) samples were collected from male. Outpatient constituted 260 (73.2%) and 95 (26.8%) samples from inpatient.

Table 1: Distribution of 355 clinical samples according to the type, gender and hospitalization

Type of sample	Gender		Total number	Hospitalization		Total number
	Male	Female		Outpatient	Inpatient	
Ear	90	70	160(45.1%)	120	40	160(45.1%)
Burn	55	50	105(29.6%)	80	25	105(29.6%)
Urine	50	40	90(25.4%)	60	30	90(25.4%)
Total	195(54.9%)	160(45.1%)	355	260(73.2%)	95(26.8%)	355

Only 65 (18.3%) isolates were belonging to *P. aeruginosa* in which 25% of them were isolated from ear swab while 16.2% and 8.9% were isolated from burn and urine respectively.

Table 2: Incidence of the isolated microorganisms in different clinical sample sites

Source of samples	No. of samples	No.(%) of *P. aeruginosa* isolates	No.(%) of Gram positive and negative isolates	No.(%) of no growth or contaminated cultures
Ear	160	40 (25)	50 (31.3)	70 (43.7)
Burn wound	105	17 (16.2)	38 (36.2)	50 (47.6)
Urine	90	8 (8.9)	24 (26.7)	58 (64.4)
Total	355	65 (18.3)	112 (31.6)	178 (50.1)

A significant differences in number of isolates were found between male and female

Table 3: Distributions of 65 *P. aeruginosa* isolates based on age, gender and hospitalization

Patients profile with positive *P. aeruginosa*	Status	No. (%) of *P. aeruginosa* isolates (n=65)
Age group (years)	(1-20)	11 (17.0)
	(21-40)	31 (47.7)
	(41-60)	13 (20)
	(over 61)	10 (15.3)
Gender	L.S.D. (0.05)	(4.7)
	Male	26(40)
	Female	39(60)*
Hospitalization	Outpatient	40 (61.5)*
	Inpatient	25 (38.5)

*** T test is significant in P<0.05**

The isolated bacteria showed variable results of resistance to aminoglycosides: gentamicin, amikacin, netilmicin and tobramycin. Based on the results from susceptibility testing, 29 (44.6%) of 65 *P. aeruginosa* isolates were found to be resistant to at least one of aminoglycosides, comprising 30 isolates (46.2%) susceptible and 6 (9.2%) with intermediate susceptibility.

Table 4: Incidence of antibiotic resistance in *P. aeruginosa* isolates (n= 65)

Antibiotic	Sensitive	%	Intermediate	%	Resistant	%
Amikacin	**52**	**80.0**	**1**	**1.5**	**12**	**18.5**
Gentamicin	**30**	**46.2**	**6**	**9.2**	**29**	**44.6**
Netilmicin	**45**	**69.2**	**7**	**10.8**	**13**	**20**
Tobramycin	**39**	**60**	**5**	**7.7**	**21**	**32.3**

To investigate the mechanism of aminoglycosides resistance among *P. aeruginosa*. Table (5) showed the distribution of aminoglycosides resistance genes among aminoglycoside resistance *P. aeruginosa* isolates. The results showed the presence of *aac(6')-Ib*in 21 (%) isolate of aminoglycoside resistance isolates. Figure (2 a and b).

Table 5: Distribution of various aminoglycosides-resistance genes and their combinations and aminoglycosides resistant profiles in AACs carried *P. aeruginosa* isolates (n=21)

Occurrence of gene	No.(%) of isolates	Isolate code No.	Aminoglycosides resistant profile
aac(6')-Ib	**10 (34.4)**	**26, 28, 29, 63**	**CN**
		38, 57	**AK, CN, TOB**
		42, 52	**CN, TOB**
		13	**CN, NET, TOB**
		64	**AK, CN, NET, TOB**
aac(6')-Ib + aac(6')-I	**5(17.2)**	**11, 24, 25**	**CN, NET, TOB**
		21, 35	**AK, CN, NET, TOB**
aac(6')-Ib + aac(3)-II	**2 (6.9)**	**23**	**CN, NET, TOB**
		46	**CN**
aac(6')-Ib + aac(3)-I	**1 (3.5**	**17**	**AK, CN, NET, TOB**
aac(3)-I	**1 (3.5)**	**15**	**AK, CN, NET, TOB**
aac(6')-Ib+ aac(6')-I + aac(3)-II	**1 (3.5)**	**40**	**AK, CN, TOB**
aac(6')-Ib+ aac(6')-I + aac(3)-I	**1 (3.5)**	**19**	**CN, NET, TOB**
AME and *16rRNA* methylase genes negative Isolates	**8 (27.6%)**	**1, 45**	**AK, CN, TOB**
		7, 16, 58, 62	**CN**
		9, 12	**AK, CN, NET, TOB**

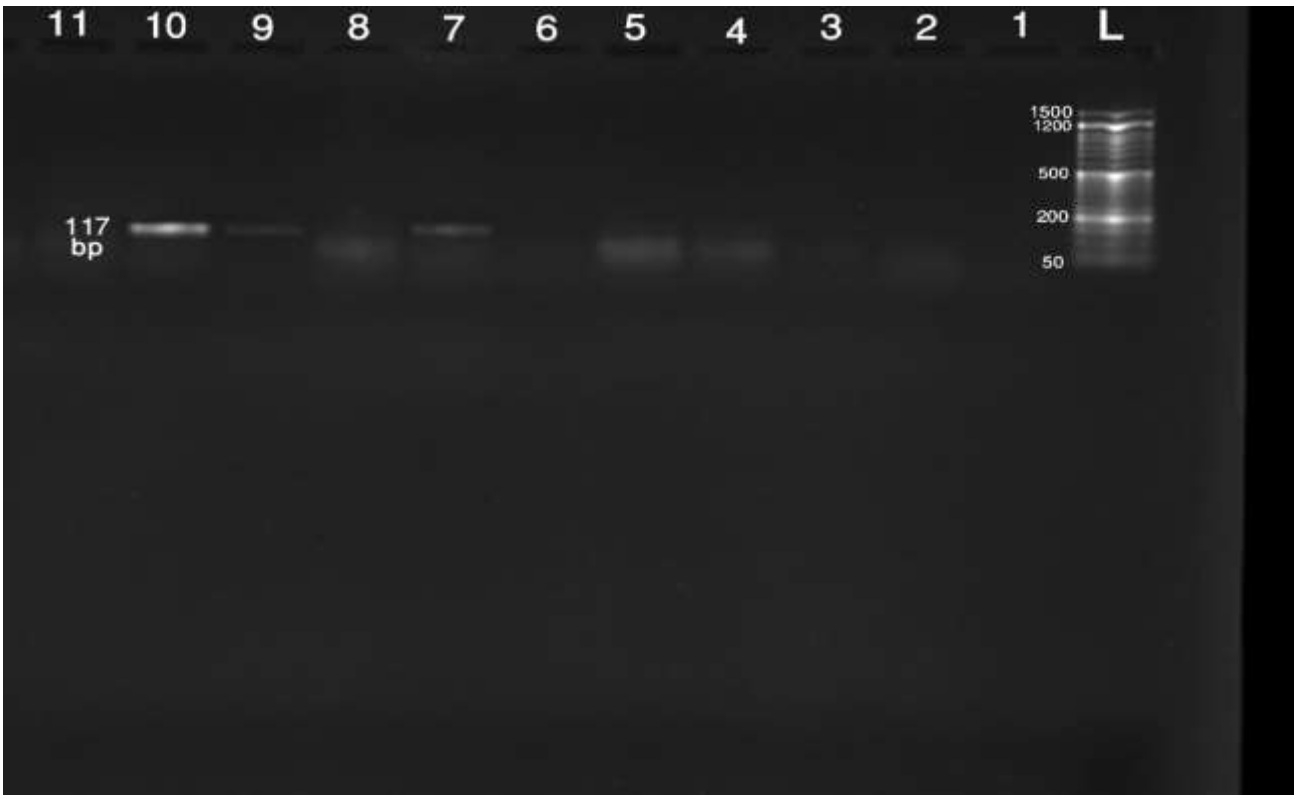
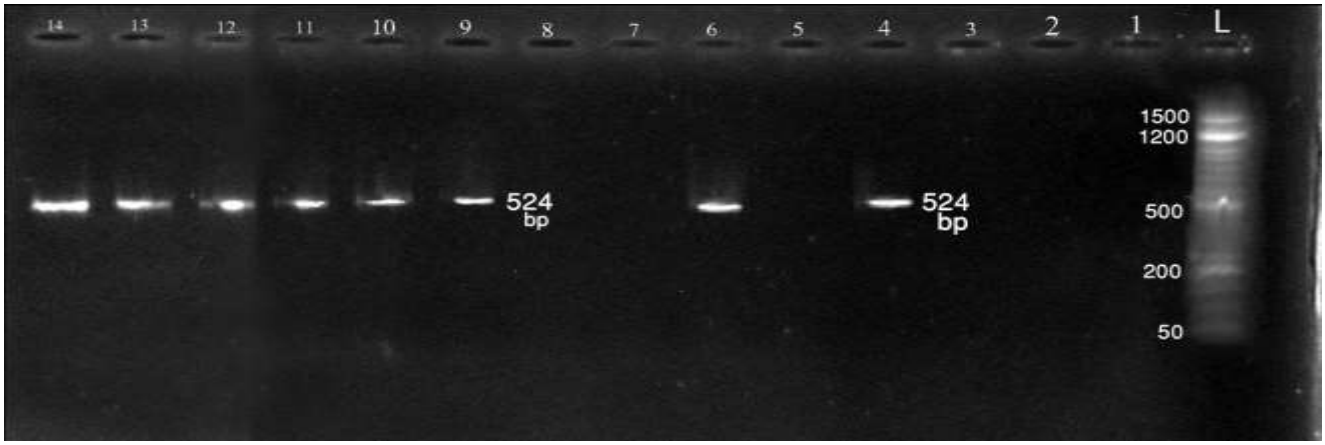


Figure (1): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *Pseudomonas aeruginosa* amplified with *aac(3)-I* gene primers. The electrophoresis performed at 60 volt for 2hr. Lane (L), DNA molecular size marker (1500-bp ladder). Lanes (7, 9, 10) of isolates show positive results with *aac(3)-I* (117 bp).



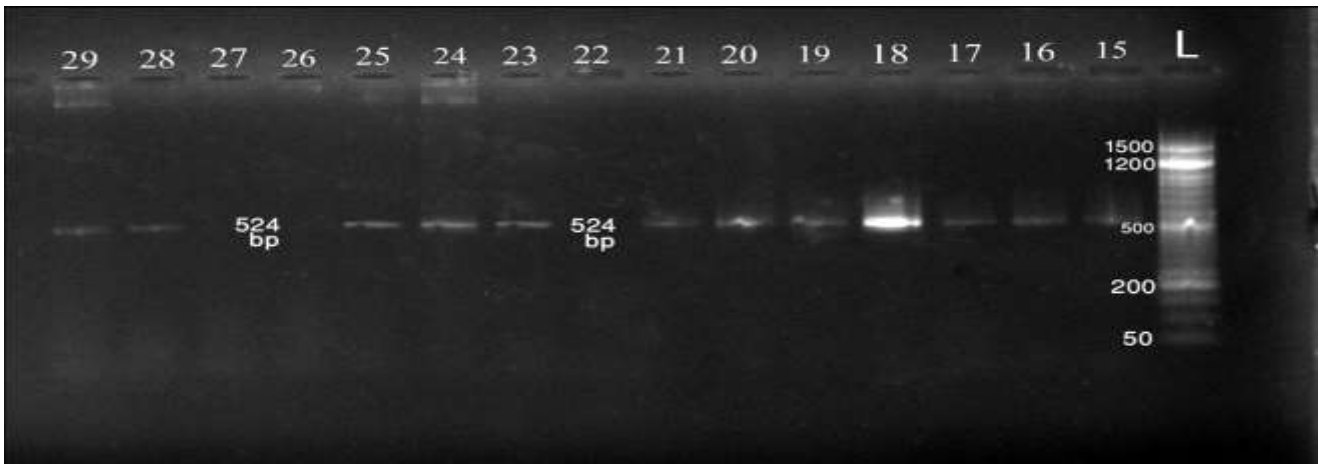
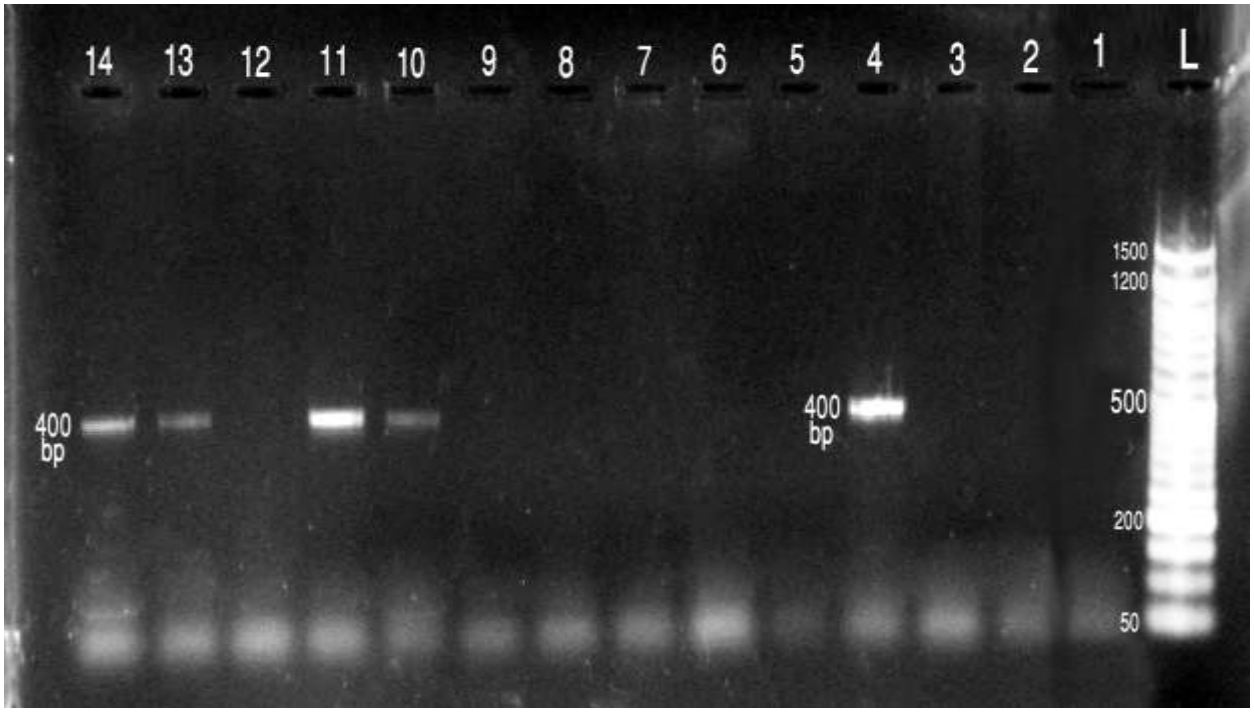


Figure (2 a and b): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *Pseudomonas aeruginosa* amplified with *aac(6')-Ib* gene primers. The

electrophoresis performed at 60 volt for 2 hr. Lane (L), DNA molecular size marker (1500-bp ladder). Lanes (4 , 6 , 9 , 10 , 11 , 12 , 13 , 14 , 15 , 16 , 17 , 18 , 19 , 20 , 21 , 23 , 24 , 25 , 28 , 29) of isolates show positive results with *aac(6')-Ib* (524 bp).



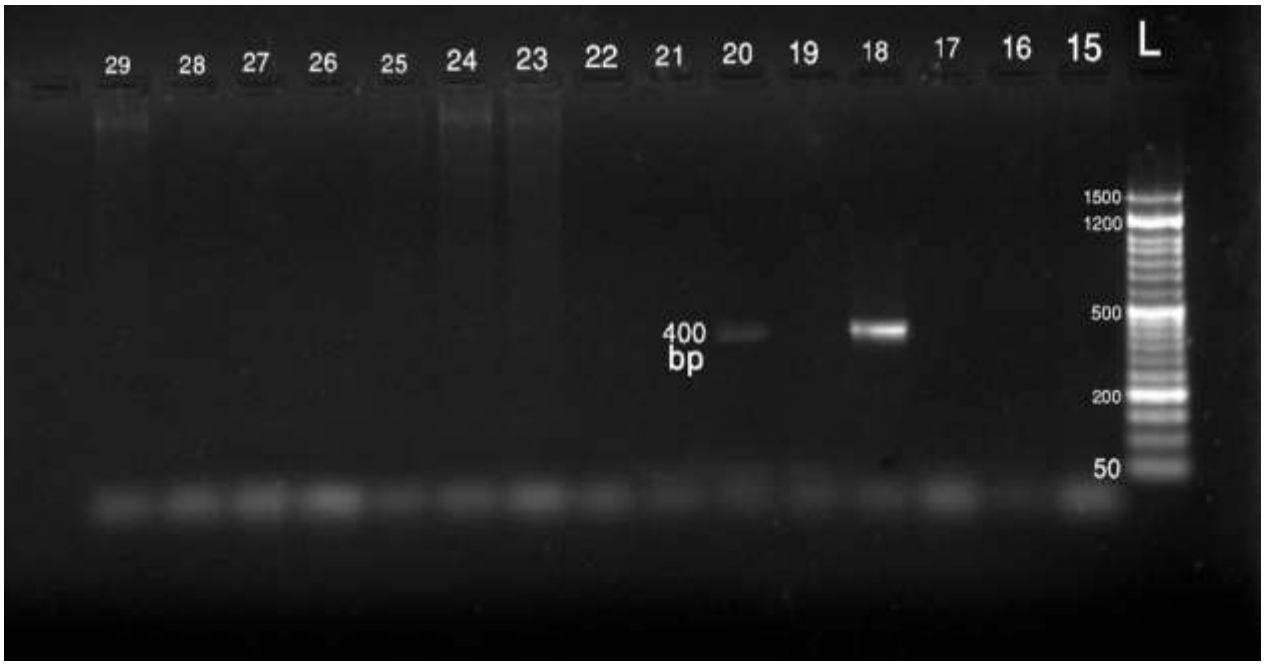


Figure (3 a and b): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *Pseudomonas aeruginosa* amplified with *aac(6')-I* gene primers. The electrophoresis performed at 60 volt for 2 hr. Lane (L), DNA molecular size marker (1500-bp ladder). Lanes (4 , 10 , 11 , 13 , 14 , 18 , 20) of isolates show positive results with *aac(6')-I* (400bp).

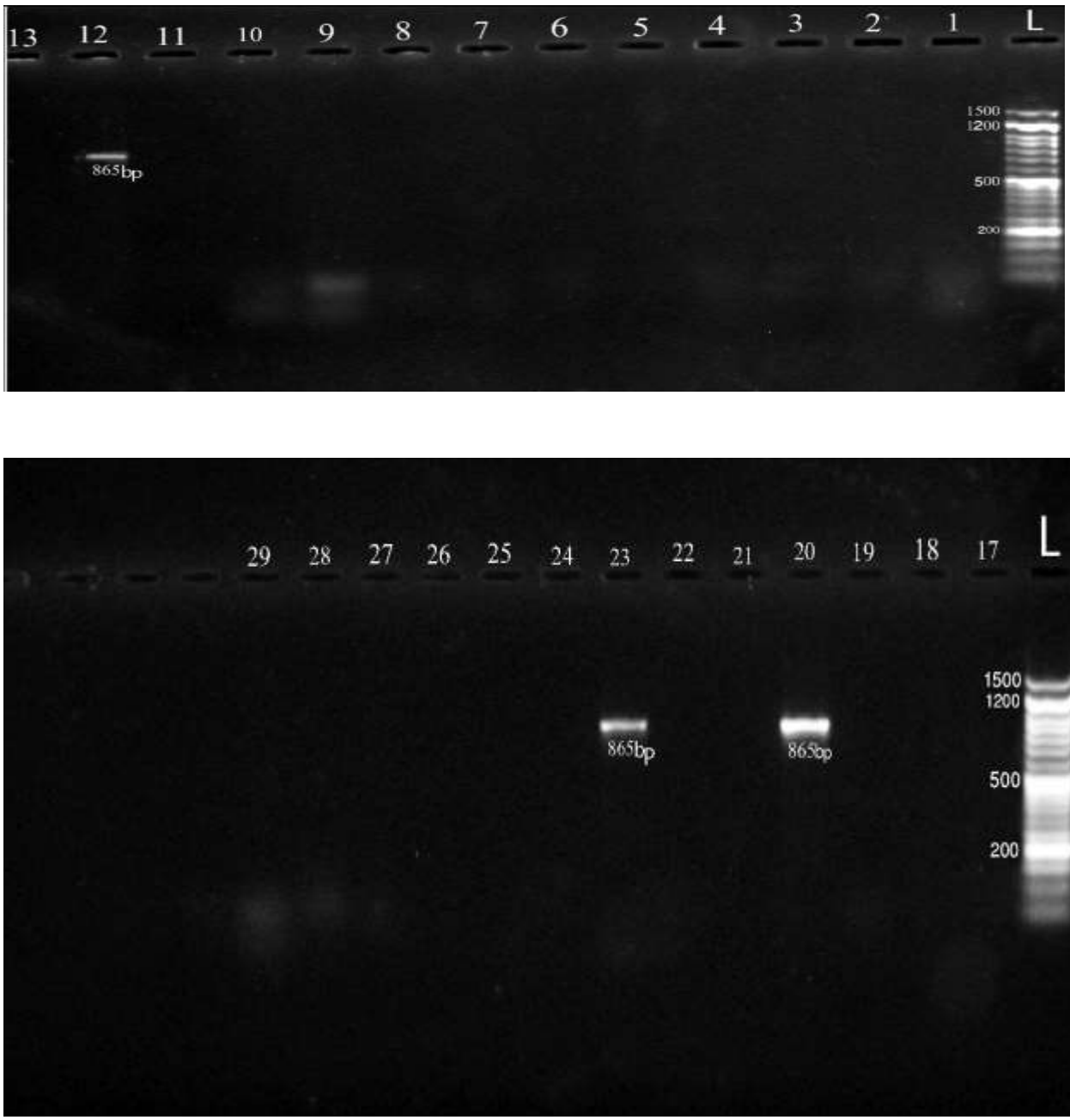


Figure (4 a and b): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *Pseudomonas aeruginosa* amplified with *aac(3)-II* gene primers. The electrophoresis performed at 60 volt for 2 hr. Lane (L), DNA molecular size marker (1500-bp ladder). Lanes (12, 20, 23) of isolates show positive results with *aac(3)-II* (865 bp).

DISCUSSION

The incidence of *P. aeruginosa* among the examined samples was 65 positive isolates with a percentage of 18.3%. This incidence corresponds with many publications in Iraq [14, 15, 16]. The present investigation found that *P. aeruginosa* was most commonly isolated (25%) from the ear infection and this consistent with some previous surveillance studies [17, 9]. Previous studies performed in burn hospitals in various cities of Iraq [15, 10], demonstrated high incidence of these isolates. The results of the present study showed that out of the entire burn culture positive, 16.2% exhibited *P. aeruginosa* isolates. Out of 90 patients with UTI, 8.9% patients had an established *P. aeruginosa* etiology. This rate was comparable with study recorded 9.8% *P. aeruginosa* among urine infection in Najaf [11].

This study has focused on resistance to four types of aminoglycosides in clinically isolates of *P. aeruginosa* from hospitals, with emphasis on gentamicin, tobramycin, netilmicin and amikacin. In this study the percentage of resistance to gentamicin (44.6%) was low when evaluated with previous

reports published from Iraqi provinces, 74.6-87% [18, 10]. Interestingly, percentage of resistance to tobramycin (32.3%) was less than previous reports (62.2-77.1%) published in other studies [10, 11, 19]. There are no available national surveillance data regarding the occurrence of resistance to netilmicin in Iraq. Present study reveals low incidence of resistance to netilmicin among the clinical isolates of *P. aeruginosa* (20%). Amikacin demonstrated excellent activity against *P. aeruginosa* isolates (80.0% susceptibility), which is consistent with results from other studies in Iraq [18, 15]. The high activity of amikacin may be attributed to the presence of the aminohydroxybutyryl group, which generally prevents the enzymatic modification of amikacin at multiple positions without interfering with binding to the A site of rRNA [20].

In the present study the aminoglycoside resistance rate in 29 isolates of *P. aeruginosa* was high (72.4%). Most of resistant isolates harbored at least one of AACs gene. The *aac(6')-Ib* and *aac(6')-I* were the most common detected AAC (69% and 24.1%, respectively). These results are similar to that has been observed in different studies in other countries [21, 22]. Though AAC(3)-II has been reported to be rare enzyme in *P. aeruginosa*[23], this was present in 10.3% of the isolates in the present study. Interestingly, ten of aminoglycoside-resistant isolates had a combination of two to three different resistance genes, and five different combinations were encountered, which is in contrast to several studies conducted in the USA and Europe, which reported that the majority of isolates exhibit only a single aminoglycoside modifying gene [22]. The difference in the distribution of modifying enzymes may derive from differences in aminoglycoside prescription patterns, the selection of bacterial population or geographical differences in the occurrence of aminoglycoside resistance genes. Results revealed that all of the isolates tested were found to be negative in the PCRs technique for the presence *16rRNA* methylase genes, *aph(3')-VI* and *ant(4')-IIb*. The results support the hypothesis that AACs is representing the dominant aminoglycosides resistant type in *P. aeruginosa* isolates.

The 21 of 29 aminoglycosides resistant isolates carrying plasmid-borne *aac* genes revealed unexpected resistance phenotypes. For example, when an isolate harbored only the *aac (3)-I* gene (isolate no. 15) which confer resistance to gentamicin, tobramycin and netilmicin but exhibited susceptibility to amikacin, resistance to amikacin, gentamicin, tobramycin and netilmicin was observed. We presume that the reason for this phenomenon might be the action of other resistance mechanisms, such as impermeability, efflux pumps, or other types of modifying enzymes.[4]Interestingly, the current investigation did not discover any of resistant genes to aminoglycosides in 8 isolates in spite of the presence of the aminoglycosides resistance phenotypes. The present study speculate that other resistance mechanisms including the reduction of antibiotic penetration on the outer membrane protein, and augmented excretion by an efflux pump system may exist[8]. However, compared with the aminoglycosides susceptibility results, there was no evident correlation between the presence of a particular AAC enzyme and the resistance pattern and this finding needs further investigation.

CONCLUSIONS:

The aminoglycosides resistance rates in *P. aeruginosa* were roughly high in Al-Nasseryia, and most of the resistance isolates harbored *aac (6')-Ib* gene.

RECOMMENDATIONS:

In order to overcome the worrisome development of increased resistance to antibiotics in general and aminoglycoside in particular, continued national surveillance programs are crucial.

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