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Molecular Characterization Aminoglycosids Resistance *Pseudomonas aeruginosa*

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Abstract

Resistance to aminoglycosids is a great problem to therapeutics. Aminoglycoside acetyltransferase producing *Pseudomonas aeruginosa* have been reported to be important cause of nosocomial infections. The purpose of this study was to determine the occurrence of aminoglycoside acetyltransferase. A total of 200 clinical and environmental samples were collected over period of five months. The *P. aeruginosa* isolates were confirm their identification, antibiotic susceptibility profile according to vitek2 compact system. The isolates were subjected to polymerase chain reaction (PCR) assays with specific primers for aac (6')-I, aac (6')-Ib, aac (3')-I . Only 32 (16.%) *P. aeruginosa* isolates were recovered from the samples. in present investigation. Gentamicin seemed to offer more resistance (31.3%) than tobramycin (28.1%), which itself is slightly more resistance than amikacin (25%).In PCR experiments using specific primers for genes. *aac(6')-I* , and *aac(6')-Ib* were present in 12 (37.5%) and 15 (46.9%) of the isolates, respectively. While the *aac (3')-I* were negative among all isolates.

Keywords: Aminoglycosids, occurrence of aminoglycoside acetyltransferase.

التشخيص الوراثي للزوائف الزنجارية المقاومة للمضادات الامينوكلايكوسيدية

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الخلاصة:

تعتبر صفة المقاومة للامينوكلايكوسيدات مشكلة علاجية كبيرة في بكتريا الزوائف الزنجارية المنتجه لانزيمات الاستيل ترانسفريزالكلايكوسيدية ، حيث اعتبرت احد الاسباب المهمة للاصابات المكتسبة من محيط المستشفى. الغرض من هذا البحث هو تحديد مدى انتشار انزيمات الاستيل ترانسفريزالكلايكوسيدية. جمعت 200 عينه (110 عينه سريرية ، 90 عينه من محيط مستشفى الصدر) لفترة امتدت لمدة خمسة اشهر . شخصت العزلات الزوائف الزنجارية واختبرت حساسيتها الدوائية بواسطة جهاز الفايتك . تم الحصول على 32 (16%) عزلة زوائف زنجارية من مجموع العينات .اظهرت النتائج ان المقاومة للجنتامايسين (31.1%) اعلى من المقاومة التوبرامايسين (28.1%) الذي بدوره اعلى مقاومة من الاميكاسين (25%) . استعمل تفاعل سلسلة البلمرة لتحديد الجينات المشفرة لصفة المقاومة للامينوكلايكوسيدات بالاعتماد على بوادئ متخصصة وقد اظهرت النتائج وجود جين *aac(6')-I* في 12 عزلة (37.5%) ، و جين *aac(6')-Ib* في 15 عزلة (46.9%) أما جين *aac(3')-I* لم يظهر في أي عزلة.

Introduction

Aminoglycoside antibiotics are positively charged carbohydrate-containing molecules that have proven to be instrumental in the treatment of infectious diseases since their discovery in the mid-1940s, it use in the treatment of infections caused by both Gram-positive and Gram-negative bacteria

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and, in addition, some protozoa[1]. These antibiotics are natural products, derived from bacterial producers [2].

Streptomycin was the first aminoglycoside that identified and characterized by Waksman's laboratory from soil bacteria *Streptomyces griseus* in 1944 [3,4] Several years later, additional aminoglycosides were characterized from other *Streptomyces* species; neomycin and kanamycin in 1949 and 1957, respectively. In the 1960s, gentamicin was recovered from the actinomycete *Micromonos porapurpurea*. Because most aminoglycosides have been isolated from either *Streptomyces* genus or *Micromonospora* genus , a nomenclature system has been set up based on their source[5,6] .

Aminoglycosides are a complex family of compounds and the classification can be based on the chemical structure. There are different structural classes of aminoglycosides, characterized by having an aminocyclitol nucleus [streptamine, 2 deoxystreptamine (DOS) or streptidine] linked to amino sugars through glycosidic bonds [7] .There are two main classes of aminoglycosides; the streptomycin class (I) and the 2-deoxystreptamine class (II) [8].

The largest group of aminoglycosides , contains several antibiotics that are clinically important for the treatment of serious Gram-negative infections (gentamicin, tobramycin, amikacin) and the third class 4-monosubstituted 2-DOS compounds are represent by apramycin, an aminoglycoside that is used only for veterinarian purposes [9]. Aminoglycoside of these three groups, 4, 5- and 4, 6-disubstituted 2-DOS derivatives and 4- monosubstituted 2-DOS compounds (apramycin), share in common a target site at the decoding center (A-site) of bacterial 16S ribosomal RNA(rRNA) [8].

Materials and Methods:

1- Materials:

Table 1-Materials used in this study.

No.	Materials	Company	Origin
1	cultures media	Himedia,	India
		ChroMOagar	France
2	reagents	Promega	USA
3	solutions	Promega	USA
4	antibiotics susceptibility test kit	BioMerieux	France
5	PCR kit	Kappa	USA
6	gene primers (*aac (3')-I, aac(6')-I* , and *aac(6')-Ib*)	Biocorp	Canada

2- Methods: All methods of cultural, cellular and biochemical tests were used according [10 , 11] . The definitive identification of *P. aeruginosa* , antibiotic susceptibility assays and minimum inhibitory concentration (MIC) determined via VITEK 2-Compact System .

Antibiotics

Table 2- Antibiotics used in this study.

Class	**Antibiotic**	**Symbol**	**Concentration(μg)**		
Aminoglycosides	Amikacin	AN	8	16	64
	Gentamicin	GM	4	16	32
	Tobramycin	TM	8	16	64

1 . DNA extraction

Extraction of DNA from bacterial cells was performed by boiling method [12]. with some modification as follows: A loopfull of *P. aeruginosa* overnight growth were inoculated in 5 ml Luria-Bertani broth and incubated at 37C° for 24 hr. 1 ml of bacterial culture transferred in to micro tube then centrifuged at 10000 xg for 10 min. The resulting pellet was re-suspended in 100 µl of TE buffer, boiled for 10 min followed by immediate chilling on ice. After its centrifugation at 10000 xg for 10 min.

2. Preparing the Primers

The DNA primers were re-suspended by dissolving the lyophilized product after spinning down briefly with TE buffer molecular grade depending on manufacturer instruction as stock suspension. Working primer tube was prepared by diluted with TE buffer molecular grad. The final picomoles depended on the procedure of each primer.

3. Primers

The following primers Table-3 were used to identify the target genes in *P. aeruginosa* isolates.

4. PCR programs

The optimization condition for PCR program Table-3, -5 were done according to [Kim, Park]

5. Agarose Gel Electrophoresis

All requirements, technical and preparations of agarose gel electrophoresis for DNA detection and analysis performed by [13].

Table 3- Target genes and primers used in this study.

Primer	Gene Name	Oligo sequence	Product size (bp)	Company	Reference
*aac(3')-I	***aac(3')-I***	**F**:5'-AGC CCG CAT GGA TTT GA-3' **R**:5'- GGC ATA CGG GAA GAA GT-3'	**117**	Biocorp Canada	[14]
aac(6')-I	***aac(6')-I***	**F**:5'-CGC GCG GAT CCC ACA CTG CGC CTC ATG A-3' **R**:5'-GAC GGG TCG TTT GAA TTC TGG TG-3'	**400**	Biocorp Canada	[14]
aac(6')-Ib	***aac(6')-Ib***	**F**:5'-TTGCGATGCTCTATGAGTGGCTA-3' **R**:5'-CTCGAATGCCTGGCGTGTTT -3	**482**	Biocorp Canada	[15]

*** aac= aminoglycoside acetyltransferase**

Table 4-The PCR mixture reaction

PCR reaction mixture	Kapa protocol (final volume 20µl)
2X Ready mix with Mg^{2+}	10µl
Primer forward (10µM)	0.8 µl
Primer reverse (10µM)	0.8 µl
DNA (100µg/ml)	4 µl
PCR grade water	4.4 µl

Table 5-PCR programs

Gene name	Temperature (C^o)/ min					Cycle number	Reference
	Initial Denaturation	Cycling condition			Final extension		
		denaturation	annealing	extension			
	(C^o)/ min						
aac(3')I	94/ 15	94/1	55/1	72/1	72/10	30	[14]
aac(6')I	94/ 15	94/0.75	55/0.75	72/0.75	72/10	34	[14]
aac(6')Ib	94/4	94/0.75	55/0.75	72/0.75	72/10	34	[15]

Results and Discussion

In this study A total of 200 clinical and environmental samples were collected over period of five months only 32 (16.%) *P. aeruginosa* isolates were recovered from the samples., all *P. aeruginosa* isolates (32 isolates) were screened against aminoglycosids antibiotics. The resistance results were determined by using VITIKE2 system as presented in Table-6. There is no typical profile for aminoglycosides resistant among isolates in present investigation. Gentamicin seemed to offer more resistance (31.3%) than tobramycin (28.1%), which itself is slightly more resistance than amikacin (25%). The resistance rate was relatively constant for Tobramycin when compared to previous reports in Iraq [16, 17] .The isolated 32 *P. aeruginosa* showed variable degrees of susceptibility to aminoglycosides as assessed by automated VITEK-2 compact system. Based on the results, the 32 isolates were separated into four groups.

First group, 8 (25%) isolates exhibited resistance to all aminoglycosides tested (gentamicin, tobramycin and amikacin). Second group, 1 (3.1%) isolate expressed resistant to gentamicin but susceptible to tobramycin and intermediate to amikacin. Third group, 1 (3.1%) isolate demonstrated resistant to gentamicin and tobramycin but susceptible to amikacin. Finally, fourth group, 22 (68.8%) isolates susceptible to all aminoglycosides tested Figure-1. All of the 32 isolates were screened for the present of aminoglycoside acetyltransferases [*aac(3)-I, aac(6')-I , aac(6')-Ib*] encoding genes. Aminoglycoside acetyltransferases genes including *aac(6')-I* , and *aac(6')-Ib* were present in 12 (37.5%) and 15 (46.9%) of the isolates, respectively Figure-2, Figure-3. No isolates were positive for the gene *aac(3)-I*. However, the combination of *aac(6')-I*+ *aac(6')-Ib* were detected in 12 (37.5%) isolates. This finding is in accordance to study conducted in Al-Nasseryia, where the *aac(6')-I* and *aac(6')-Ib* genes were the most frequent, and most of the *aac(6')-Ib* gene was present as a single[16].

In Belgium, France, and Greece, where amikacin has been used more extensively than in other European countries, the incidence of *aac(6')-I*, an enzyme that produces resistance to amikacin, was also much higher [18] In this study, one of the *aac(6')-I* and *aac(6´)-Ib* harboring isolates was susceptible to amikacin (according to Clinical Laboratory Standards Institute breakpoints) but remained resistant to gentamicin. Additionally, 6 of the 15 *aac(6´)-Ib* and/or *aac(6')-I* harboring isolates were susceptible to all aminoglycosides tested. Compared with the antibiotic susceptibility test results, the PCR results did not correlate well. It was noticed that *aac(6')-I* and *aac(6´)-Ib* confers resistance to amikacin, tobramycin, kanamycin, netilmicin, and sisomicin but not to gentamicin [19] .

However, in *P. aeruginosa*, the presence of the *aac(6')-Ib* gene may be also associated with a decreased gentamicin susceptibility [20 ، 21], studied naturally occurring variants of *aac(6´)-Ib* that had a serine residue instead of a leucine residue at position 119, change from leucine to serine at this position conferred an altered substrate profile, where the mutant *aac(6´)-Ib* conferred resistance to gentamicin instead of the amikacin resistance seen in the wild-type enzyme. Moreover, it has been demonstrated that modifications of the amino acid sequence of the *aac(6')-I* coding gene influence their enzymatic activities thus, different aminoglycosides resistant phenotypes might be observed with the same gene, and molecular techniques assayed in this investigation are indispensable to elucidate the resistance mechanisms.

Furthermore, in *P. aeruginosa*, owing to the combination of several mechanisms and variable levels of their expression, the involved mechanisms cannot be easily inferred from the resistance profiles [20]. However, a recent studies in Al-Nasiriya and Al-Najaf reported by [16,17] also described these findings.

Table 6- Antibiotic susceptibility results as determined by using VITIKE2 test.

Isolate symbol	Sample source	Amikacin	Gentamicin	Tobramycin
p1	S.W	S	S	S
p2	B.W	S	S	S
p3	B.W	S	S	S
p4	B.W	S	S	S
p5	B.W	S	S	S
p6	B.W	S	S	S
p7	B.W	S	S	S
p8	B.W	I	R	S
p9	B.W	S	S	S
p10	B.W	R	R	R
p11	B.W	R	R	R
p12	I.N	S	R	R
p13	B.W	S	S	S
p14	B.W	S	S	S
p15	B.W	S	S	S
p16	B.W	R	R	R
p17	B.W	S	S	S
p18	B.W	S	S	S
p19	B.W	R	R	R
p20	S.W	S	S	S
p21	S.W	S	S	S
p22	D.I	S	S	S
p23	F.L	S	S	S
p24	I.N	S	S	S
p25	B.W	S	S	S
p26	B.W	S	S	S
p27	B.W	R	R	R
p28	B.W	R	R	R
p29	B.W	R	R	R
p30	B.W	R	R	R
p31	B.W	S	S	S
p32	F.L	S	S	S
total	S%	71.9	68.7	71.9
	R%	25	31.3	28.1
	I%	3.1	0	0

S.W: surgical wound, B.W: burn wound, I.N: instruments, D.I: disinfectant, F.L: flour

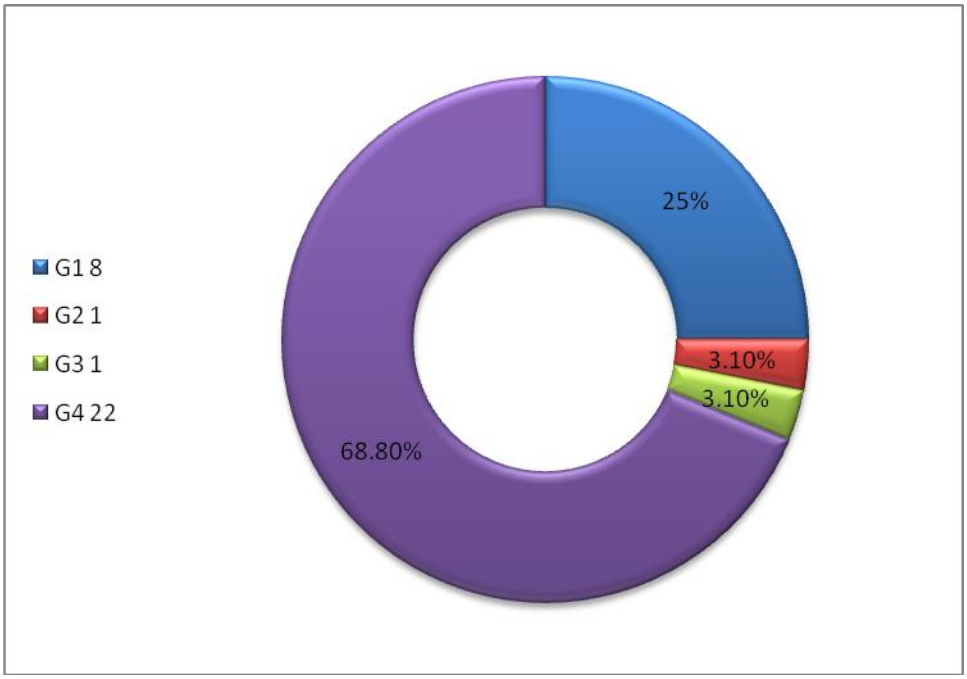

Figure 1-Aminoglycosides resistance profiles of 32 *P. aeruginosa* isolates. G1, resistant to all aminoglycosides; G2, resistant to gentamicin but susceptible to tobramycin and intermediate to amikacin; G3, resistant to gentamicin and tobramycin but susceptible to amikacin; G4, susceptible to all aminoglycosides.

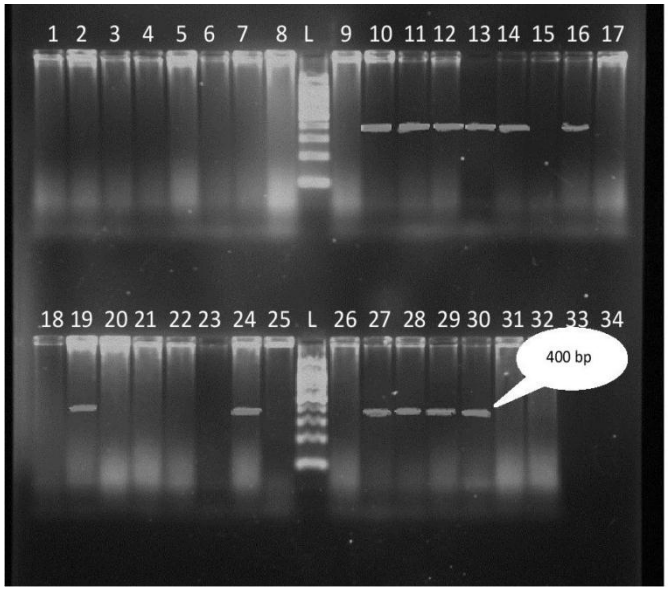

Figure 2- PCR the amplification products of *P. aeruginosa* isolates that amplified with aac(6')-I gene primer with size product 400bp. Lane (L), DNA molecular marker (1500-100 bp) ladder. Lanes (10,11,12,13,14,16,19, 24,27,28,29,30) show positive results with *aac(6')-I* gene on agarose gel (1.5%)at 60 volt for(1.5-2) hours..

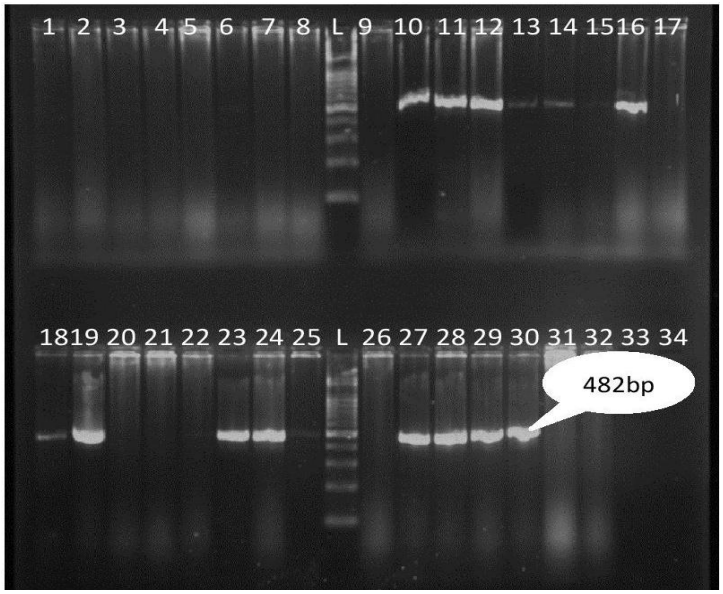

Figure 3- PCR amplification products of *P. aeruginosa* isolates that amplified with aac(6')-Ib gene primer with product size 482bp. Lane (L), DNA molecular marker (1500-100 bp) ladder. Lanes(10,11,12,13,14,16,18,19,23,24,25,27,28,29,30) show positive results with *aac(6')-I* gene on agarose gel (1.5%) at 60 volt for (1.5-2) hours.

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