Molecular Detection of Aminoglycoside Resistance Mediated by Efflux Pump and Modifying Enzymes in *Pseudomonas Aeruginosa* Isolated From Iraqi Hospitals

Sawsan Sajid Al-Jubori*, Heba Ameen Al-Jabiri, and Israa M.S. Al-Kadmy

***Abstract*—**Clinical isolates (50) of *Pseudomonas aeruginosa* were collected from patients suffering various infections at different teaching hospital in Baghdad\ Iraq. These isolates were diagnosed using the API 20E followed by genotypic detection by a housekeeping gene (*rpsI*). Results of aminoglycoside resistance using the two fold serials dilution method revealed that all the isolates conferring multidrug resistance and the highest resistance were against kanamycin followed by gentamicin , while the lowest was against amikacin. Highly resistant *P.aeruginosa* isolates (28) were selected to determine aminoglycoside resistance mediated by *mexXY* efflux system and Aminoglycosides Modifying Enzyme (AME) using polymerase chain reaction (PCR) . Out of 28 *P.aeruginosa* isolates, 25(89.5%) and 8(28%) gave positive results for efflux system(*mexX* and *mexY* genes respectively) ,while 21(75%) of the isolates harbored one or more of AME genes and the most frequent one was *aac(3)-I* which detected in 18 isolates (64.2%) followed by *aac(6)-Ib, ant(4)IIb, and aph(3)-VI* when the rates reached 12(42.8%), 8(28.5%) and 2(7.1%) respectively .This may indicate the prevalence of these types of resistance in the current isolated bacteria.

Keywords*—Pseudomonas aeruginosa*, Aminoglycoside resistance, Efflux system, Aminoglycoside modifying enzymes, *mexXY*.

I. Introduction

Pseudomonas aeruginosa is one of the most prevalent nosocomial pathogens associated with higher mortality rates and antibiotic costs(1). It is also considered as the most opportunistic human pathogen especially in immune compromised patients and one of the top five pathogens of nosocomial diseases worldwide (2).Aminoglycosides (AG) are bactericidal antibiotics that are widely used in treatment for severe infections diseases caused by Gram-negative and Gram-positive bacteria (3). Aminoglycoside molecules bind to the bacterial 30S ribosomal subunit rendering them unavailable for translation then cell death. Several aminoglycoside resistance mechanisms have been recognized in different bacterial species including (I) active efflux system (II) Inactivation of the drugs by aminoglycoside modifying enzymes(III) alteration Ribosomal target sit (IV) Decreased permeability barrier (4). *P. aeruginosa* expresses several type of multidrug efflux systems (*MexABOprM, MexCD-OprJ, MexEF-OprN*, and *MexXY-OprM*),and they are reported to be significant determinants of multidrug resistance in most clinical isolates (5).The drug ribosome interaction as a process is required for *mexXY* induction, as antimicrobials exported by *MexXY-OprM* that do not target the ribosome was unable to induce *MexXY* expression and the induction only occurs in the presence of ribosome-inhibiting antibiotics (6). *MexXY-OprM* has a role in the resistance to aminoglycoside antibiotics (2). Aminoglycosides Modifying enzymes (AME) reduce the binding of antibiotic molecule to the ribosome caused failure in activity (7]). Three families of enzymes are identified including: aminoglycoside phosphotransferases (APHs), aminoglycoside acetyltransferases (AACs) and aminoglycoside nucleotidyltransferases (ANTs) (8). In this study we aimed to genetically detect two different AG resistance mechanisms (efflux system genes and AME genes) using PCR and studying their prevalence and the correlation with phenotypic patterns of resistance

Sawsan Sajid Al-Jubori*, Heba Ameen Al-Jabiri and Israa M.S. AL_Kadmy, Department of biology – College of science /Al –Mustansiriya University. Iraq. E.mail of corresponding author*: sawsan_sajid_ma2000@yahoo.com , israa_al_kadmy@yahoo.com.

TABLE I
THE PRIMERS USED IN THE CURRENT STUDY FOR PCR AMPLIFICATION

Primer	rimers sequences 5′----------→3′	Product s (bp)	Refere nce
rpsl-F	GCAAGCGCATGGTCGACAAGA	201	(9)
rpsl-R	CGCTGTGCTCTTGCAGGTTGTGA		
mexX-F	TGA AGG CGG CCC TGG ACA TCA GC	326	(11)
mexX-R	GAT CTG CTC GAC GCG GGT CAG CG		
mexY-F	CCGCTACAACGGCTATCCCT	250	(9)
mexY-R	AGCGGGATCGACCAGCTTTC		
aac(3)-I-F	AGCCCGCATGGATTTGA	227	(12)
aac(3)-I-R	GGCATACGGGAAGAAGT		
aac(6)-Ib-F	TTG CGA TGC TCT ATG AGT GGC TA	482	(13)
aac(6)-Ib-R	CTC GAA TGC CTG GCG TGT TT		
ant(4′) IIb-F	GACGACGACAAGGATATGGAATTGC CCAATATTATT	364	(13)
*ant(4′) IIb*R	GGAACAAGACCCGTTCAATTCAATT CATCAAGTTT		
aph(3) VI-F	TAT CTC GGC GGC GGT CGA GT	800	(14)
aph(3′)VI-R	CAC GCG GGG AAA CGC GAG AA		

II. MATERIAL AND METHODS

A. Collection clinical samples :

Clinical isolates of P.auroginosa (50 isolates)were obtained from patients suffering various infections from different teaching hospital in Baghdad\Iraq during the period between July 2012 till December 2012. These samples were distributed as [14] isolates from urinary tract infections, [9] isolates from bacteraemia [8] isolates from wounds infections, [8] isolates from otitis media, [6] pus swaps from eye infections, [4] isolates Sputum from patients suffering from respiratory tract infection and [1] isolate from fractures. Bacterial diagnosis was performed using Api 20 NE KIT, followed by the genotyping detection using PCR technique.

B. Genotyping detection for isolates

Rpsl gene(a house keeping gene) was used for bacterial diagnosis. Specific primers listed in (table1) were employed and the amplified size was 201 bp.PCR was run under the following conditions (table 2) starting with a primary denaturation step at 95°C for 5 min then 30 repeated cycles started with a denaturation step at 94°C for 30sec,then annealing at 57°C for 30sec, and 1 min at 72°C as extension step followed by final extension step at 72°C for 7 min (9).

C. Antibiotic susceptibility test

Minimum inhibition concentration (MIC)for all the isolates were determined by the two fold serial dilution methods according to the CLSI(10).The used aminoglycoside antibiotic were: amikacin,gentamicinand kanamycin.

D. Detection of mexXY-OprM genes and AME genes using PCR

The *mex X* (amplified size 326 bp),*mexY* (amplified size 250 bp) and four different aminoglycoside modifying enzyme genes(*aac(3)-I , aac(6)-Ib, ant(4)IIb, and aph(3)-* VI)were detected using specific primers listed in (table 1). The reaction mixture was prepared according to the procedure suggested by the manufacture company (KAPA, South Africa) .Template DNA was prepared by boiling 5 pure colonies in 25 µl D.W for 10 min. PCR mixture composed from 6 µl template for *aac(3)-I* and 5 µl template for other genes , 12.5 µl of Go Taq® Green Master Mix (2x) , 1.5 µl from forward and reverse primers for *mex X, mexY*and *aac(6)-Ib,* while 2µl for other genes (final concentration 10pmol), then the volume was complete to 25 µl of nuclease free water for each gene.

PCR condition were optimized by repeated changing annealing temperatures (from 43 to 62 according to primers) and number of cycles (from 30 to 35) according to this study till being fixed at the condition listed in table (2). Usually the process started with initial denaturation step (ranged from 94ºC to 96ºC for 5 min) followed by repeated cycles which consists from denaturation step (ranged from 94ºC to 96ºC), annealing step (depends on the primers) then the extension step (mostly at 72 ºC) followed by final extension step (usually at 72°C).PCR products were electrophoresed in 1.0% agarose gels and visualized under UV light according to Sambrook and Russell(15).

TABLE II
PCR REACTION CONDITION FOR THE AMPLIFIED GENES

RCR Genes	Initial denatur ation	No of cycles	Denat uratio n	Prime r Annea ling	Elo ngat ion	Final extension
rpsl	95°C / 5 min	30	94°C / 30 sec	57 °C /30 sec	72° C / 1 min	7 min/72 °C
mexX	95°C / 5 min	35	95°C / 1 min	62°C/1 min	72° C / 1 min	7 min / 72 °C
mexY	95°C / 5 min	30	94°C / 30 sec	59°C/3 0 sec	72° C / 1 min	7 min / 72 °C
aac(3)-I	94°C / 5 min	33	95°C / 1 min	43°C/3 0 sec	72° C / 1 min	10min / 72°C
aac(6)-Ib	95°C / 5 min	30	95°C / 15 sec	56°C / 1 min	72° C / 30 sec	4 min / 72 °C
ant(4′)-IIb	96°C / 5 min	30	96°C / 30 sec	57°C/3 0 sec	72° C / 30 sec	5 min / 72 °C
aph(3′)-VI	95°C / 5 min	35	94°C / 30 sec	55°C/3 0 sec	72° C / 1 min	7 min / 72 °C

III. RESULTS AND DISCUSSION

P.aeruginosa is responsible for 10–15% of the nosocomial infections worldwide. Often these infections are hard to treat due to the natural resistance of the species, as well as to its remarkable ability of acquiring further mechanisms of resistance to multiple groups of antimicrobial agents(16). In

this study, fifty isolates of *P.aeruginosa* were collected from different clinical samples. Figure (1) shows the source and percentage of these isolates.

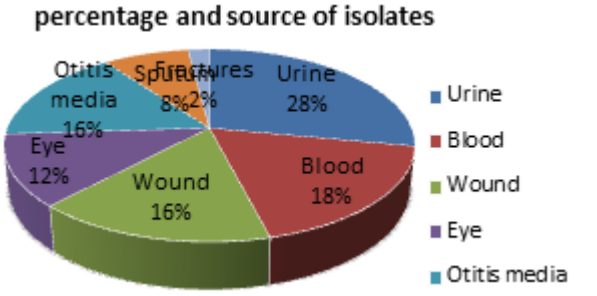


Fig 1 Distribution of *P. aeruginosa* isolates according to source of isolation

Because standard phenotypic methods are time consuming and most have inherent limitations(17), genotypic detection depending on certain housekeeping gene was used as confirmatory test and provide a rapid diagnostic technique for the identification of bacteria specially *P. aeruginosa* (18). Figure (2) shows agarose gel electrophoresis of *rpsl* PCR products for *P.aeruginosa* isolates, as it clear positive result in all lines so its indicate all isolate *P.aeruginosa* no other species of *Pseudomonas*.

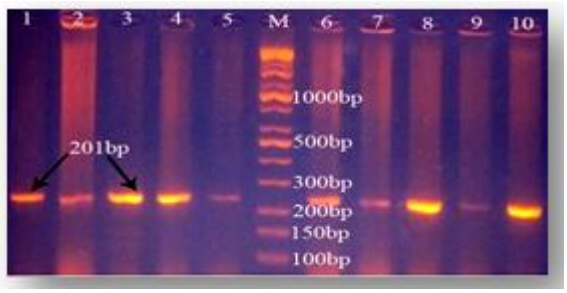


Fig 2 :Agarose gel electrophoresis electrophoresis (1% agarose, 7V/cm for 90min) of *rpsl* gene PCR product (201bp amplicon). lane M ,DNA ladder, lines 1-10 positive results

Aminoglycoside antibiotics are widely used in clinical settings, especially for treatment of life-threatening infections caused by Gram-negative bacteria(19,20). From noticing the net result obtained from MIC values,it could be said that these isolate showed very high rate of resistance to aminoglycoside antibiotic as indicated from the elevated MIC values. Factors affecting the increase and dissemination of antimicrobial resistance can be divided into transfer of resistance genes from one microbe to another and mutation of existing genes to more resistant variants by the over–use and misuse of antimicrobial (21). In the current study amikacin was the most effective one since the percentage of resistance were 30% all the isolates 100% showed their resistant to gentamicin (MIC ranged from 64–1024 µg /ml), and kanamycin (MIC ranged from 128–1024 µg /ml).Figure (3) shows a comparison between the percentage and the rang of resistance to aminoglycoside antibiotics for *P.aeruginosa*

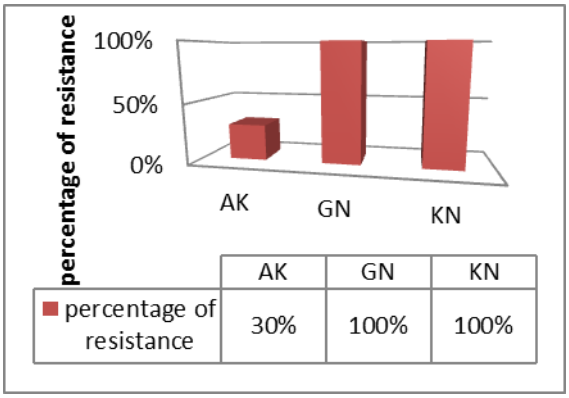


Fig. 3 The percentage of resistance of *P.aeruginosa* towards Aminoglycoside antibiotics.AK:amikacin,GN: gentamicin, KN: kanamycin

The result of the current study comparable with the results of Kim *et al*. (22) who identified 55 isolates of *P. aeruginosa* in which 22% of them were resistant to amikacin while Haldorsen (13)reported that the rate of resistance to amikacin was 30%which agree with current study. In a research done by Al-kadmy (23), reached (47%) which is a not agreement with the current study. In the research done by Dubois *et al*,.(24) they illustrated that the rate of resistancefor gentamicin reached to 55.8%and these result are much lower than the percentage of this study. On the other hand Ferguson (25) found that the rate of resistant to kanamycin 100%, while Al-kadmy (23) reported that the rate of percentage for this antibiotic was 92.8%. These results may attributed to more than one resistance mechanisms including drug inactivation due to producing modifying enzymes encoded either by plasmid- or chromosome or due to defects in uptake of antibiotic which result from impermeability resistance beside changing the target side for the antibiotic action beside the newly discovered methylation mechanism (26,27).

Twenty eight isolates were selected to detect the presence of two AG resistance mechanisms (efflux system genes and AME genes).*MexX* gene was detected in 25 (89.5%) isolates and *mexY* was detected in only 8/28 (28.5%) isolates. Figure (4) shows agarose gel electrophoresis for two efflux system genes(*mexX* and *mexY*).

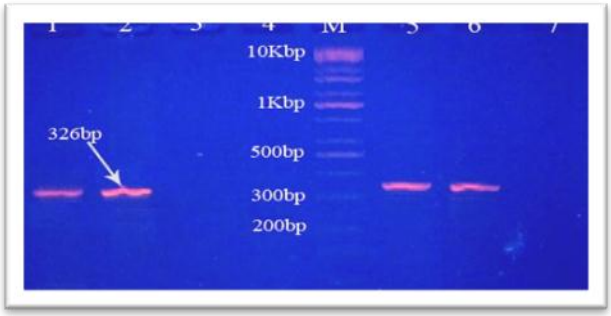


Fig.(4b): Agarose gel electrophoresis (1% agarose, 7 V/cm^2 for 90min) for *mexY* PCR products (250bp ampicon) .lane M 100bp DNA ladder. Lanes 1, 2, 3, 5, 7, 8, 9are positive results, while lanes 4, 6 show negative results

Fig (4b): Agarose gel electrophoresis (1% agarose, 7 V/cm^2 for 90min) for *mexY* PCR products (250bp ampicon) .lane M 100bp DNA ladder. Lanes 1, 2, 3, 5, 7, 8, 9are positive results, while lanes 4, 6 show negative results.

In the study of Cabot et al.(28) they showed that the prevalence rate of mexY gene without over expression was 81.5% and this result is not in line of the current study, while Ozer et al.(29) illustrated that the prevalence rate of mexX was 4% and this result not agreed with the current study. The genes coding for the modifying enzymes either present alone or in combination between two genes or three genes but can be seen none of the isolates in the current study was observed to contain the 4 detected genes. Tables (3) show the isolates harboring these genes and their percentage.

TABLE (3)
FREQUENCY OF AMES GENOTYPES AMONG BACTERIAL ISOLATES

Genes	No. of isolates out of 28(%)
Without any AMEs genes	7(25%)
aac(3)-I	18(64.2%)
aac(6)-Ib	12(42.8%)
ant(4)- IIb	8(28.5%)
aph(3)- VI	2(7.1%)
aac(3)-I+ aac(6)-Ib	6(21.4%)
aac(3)-I+ ant(4)- IIb	5(17.8%)
aac(3)-I + aac(6)-Ib + ant(4)- IIb	3(10.7%)
aac(3)-I+aac(6)-Ib+ aph(3)VI	1(3.5%)
aac(3)-I + aac(6)-Ib + ant(4)- IIb+ aph(3)VI	0(0)

The results of this study showed that the aac(3)-I was the most prevalent AME genes since it was found in 18/28 (64.2%) of the isolates followed by aac(6)-Ib,ant (4)- IIb and aph(3)-VI when the rates were 42.8% (12/28), 28.5% (8/28) and 7.1% (2/28) respectively. Figure (5) illustrates PCR products for four AME genes

Fig.(5a): Agarose gel electrophoresis (1% agarose, 7 V/cm^2 in 90min)for *aac(3)-I* gene PCR products (227bp amplicon). Lane M: 100bp DNA ladder, lanes 1,2,4,5,6,7 are positive results, while lane 3 shows negative results

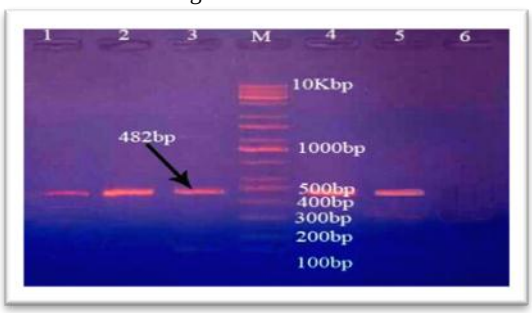


Fig.(5b): Agarose gel electrophoresis (1% agarose, 7 V/cm^2 in 90min) for *aac(6)-Ib* gene PCR products (482bp amplicons) .Lane M: 100bp DNA ladder, lanes 1,2,3,4,5 are positive results, while lane 6 is negative result

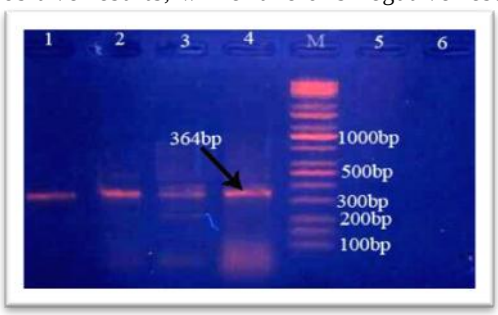
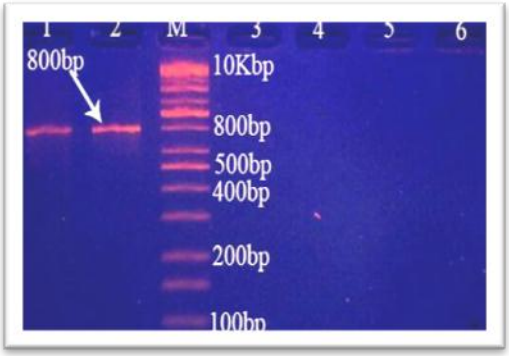


Fig.(5c): Agarose gel electrophoresis (1% agarose, 7 V/cm^2 in 90min) for *ant(4)- IIb* gene PCR products with 364bp size .Lane M: 100bp DNA ladder, lanes 1,2,3,4 are positive results, while lanes 5,6 are negative results



Fig(5d): Agarose gel electrophoresis (1% agarose, 7 V/cm^2 in 90min) for *aph(3)- VI* gene PCR products with 800bp size .Lane M: 100bp DNA ladder, lanes 1,2 as positive result, while lanes 3,4,5,6 are negative results.

The percentage of *aac(3)-I*gene in the current study is very high as compared with the results of Over *et al.*(30) and Gad *et al.*(31) when they illustrated that *aac(3)-I* gene were detected in 16.6% and 48.9% of *P.aeruginosa* isolates respectively. Also The result of current study didn't agree with Hamed *et al.*(32) who found that none of their isolates were harbouring this gene. For the second modifying genes (*aac(6)-Ib*), theresult was relatively closed with the results of Dubois *et al.* (24) in France when their rate was 36.5%, while Jafari *et al.*(33) found the prevalence rate of *aac(6)-Ib* gene reached 74% in *P.aeruginosa* ,a result which is relatively high as compared with the current ratio. While Ndegwa *et al.* (12) in Kenya and Kim *et al.*(22) in Korea illustrated that none of their isolates were harbouring *ant(4)- IIb*gene. The less percentage of last AME genes (*aph(3)- VI*)relatively close with the rate reported by Kim *et al.* (22) who showed that the prevalence of *aph(3)- VI* gene was 5.5%, while Park (34) in Korea and Vaziri *et al.*(14) in Iran reported higher percentage of *aph(3)- VI* prevalence when their rate reached 14.8% and 11% respectively.

Other results revealed that 7/28 (25%) isolates devoid any types of AME in spite of high level of their own resistance against the antibiotics. On the other hand some isolates contain more than one gene as a combination when 6/28 (21.4%) isolates have a combination between *aac(3)-I* and *aac(6)-Ib* and this combination was the most observed one . In a study carried out by Over *et al.*(30) in Turkey they found 7 (4.7%) of the isolates with elevated levels of MICs values for harboring *aac(3)*gene but in combination with *aac(6)-III* beside harboring permeability resistance genes. The second observed combination in this study was between *aac(3)-I* and *ant(4)- IIb* and it was detected in 5/28 (17.8%) isolates. Four isolates (14.2%) contain 3 AME genes as 3 (10.7%) isolates contain the combination *aac(3)-I* , *aac(6)-Ib* and *ant(4)-IIb* and one isolate (3.5%) contain the combination *aac(3)-I* , *aac(6)-Ib* and *aph(3)-VI*. Such combinations are not detect in any previously reports, while Kim *et al.*(22) detected *aac(6)-Ib + aph(3)-VI* in 6/36 (16.7%) without detecting *aac(3)-I* gene.

In the current study, it could be noticed the correlation between higher MICs values and efflux genes expression which was frequently found in combination with modifying enzymes genes. The results revealed that 67.8% of the isolates (19/28) were harboring both AMEs and efflux genes and it could be seen that most of isolates harboring one of the efflux pump genes (25 and 8 for *mexX* and *mexY* genes respectively) in corporation with one or more of AMEs. Usually aminoglycoside acetyltransferase (AAC) enzyme encoded by *aac(3)-I*and *aac(6)-Ib* genes have high activity against gentamicin specially the first gene (35). It can be noticed that all isolates harboring *aac(3)-I* gene can resist gentamicin except some of the isolates which still can resist gentamicin but devoid this gene. It is possible that the efflux system gave a good reason for this resistance because all these isolates were harboring *mexX* gene in alone orin corporation with *mexY* geneand the *aac(6)-I* gene was detected in 12 isolates and all these isolates were resistant to gentamicin . Usually AG nucleutidltransferases (ANT) and AG phosphotransferase (APH) enzymes encoded by *ant(4)-IIb* , *aph(3)-VI* respectively is known to mediated resistant to amikacin and kanamycin in *P.aeruginosa* (8). In the current study all isolates harboring the *ant (4)-IIb* gene were resistant to amikacin (MICs were between 64-128 µg/ml) and Kanamycin (MICs were 128-1024 µg/ml) as well as high elevated MICs to other type of antibiotic. Some isolates showed high phenotype resistance to amikacin, gentamycin and kanamycin aminoglycosides antibiotics without harboring either *ant(4)-IIb* nor *aph(3)-VI* and other isolates were possessing only *mexX* gene, but still resistant to all AG group except to amikacin.The reason for the discrepancy between PCR and MICs might be the action of other resistance mechanisms, such as impermeability, 16s RNA methylation or other producing types of modifying enzymes (14).

In conclusion out of 28 isolates, 27 (96.4%) were harbored at least one AG mechanism but still there were some isolates that devoid any type of the screened genes, a finding that push forward to search for other mechanism which add the bacteria to survive the antibiotics

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