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RESEARCH ARTICLE

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

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Abstract

Pseudomonas aeruginosa is one of the primary opportunistic pathogens responsible for nosocomial infections. Aminoglycosides are an important component of antipseudomonal chemotherapy. A total of 350 clinical isolates were routinely collected. The isolates were classified according to the source of collection into four groups (162 ear swab, 98 burn swab, 50 sputum samples and 40 urine samples). The results of culture and biochemical tests showed that 50 isolates were *Pseudomonas aeruginosa*, which were then confirmed by API-20E. Antibiotic susceptibility test (by using the Kirby-pour technique) was performed on all these isolates. The results of antibiotic susceptibility test showed that 32 isolates (64%) resistance to at least one type of aminoglycosides which represented by gentamicin the highest (64%) and amikacin (26%) the lowest. The isolates that resistance to at least one type of aminoglycosides were screened for the presence of modifying enzyme genes (AACs, APHs, ANTs) and 16S rRNA methylase genes by polymerase chain reaction. Five genes of AAC were found in 32 (64%) aminoglycosides resistant isolates, *aac(6')-Ib* (87.50%) was the most frequently identified gene, followed by *aac(3)-II* (31.25%), *aac(6')-I* (21.88%), *aac(3)-I* (15.63%) and *aac(6')-Iib* (8.33%). This study noted that the presence of *aph(3')-VI* (25%) and *ant(4')-Iib* (21.88%). All of the isolates tested were exhibited positive results for the presence of the 16S rRNA methylase, *armA* (6.25%) was the lowest frequently identified gene among the 16S rRNA methylase genes, *rmtA* (31.25%), *rmtD* (18.75%), *npmA* (43.75%) was the most frequently identified gene among the 16S rRNA methylase genes.

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INTRODUCTION

Pseudomonas aeruginosa is a non-fermentative Gram negative bacteria widely distributed in nature and can survive on a wide variety of surfaces and in hospital environment, as the wards encourage bacterial growth (1). Aminoglycosides are a group of clinically important, broad-spectrum antibiotics that inhibit protein biosynthesis in bacteria by selectively binding to the A-site decoding region of the bacterial 16S rRNA within the 30S ribosomal subunit causing mistranslation of mRNA or premature termination of protein synthesis (2). Resistance to aminoglycoside may be linked both with chromosomal mutations and acquisition of resistance genes located on mobile genetic elements, such as plasmids, integrons, and transposons (3). From variety of plasmid-mediated aminoglycoside resistance mechanisms, the most commonly encountered is the production of aminoglycoside-modifying enzymes (4). These modifying enzymes include aminoglycoside phosphoryl transferase (*aph*), aminoglycoside acetyl transferase (*aac*), and aminoglycoside nucleotidyl transferase (*ant*) (5). High level of resistance to aminoglycosides can also be mediated with production of 16S rRNA methyltransferases, which preclude disturbance of protein synthesis caused by aminoglycoside molecule (6). The aim of this study was to

determine the occurrence of aminoglycoside resistance and the prevalence of the 16S rRNA methylase and resistance-modifying enzyme genes in *P. aeruginosa* isolated from Al-Diwaniya teaching hospital in Al-Diwaniya province.

Materials and Methods:

Collection of Samples: During the period from November 2014 to April 2015, a total of 350 nonduplicate clinical samples were collected from patients visited/or admitted to Al-Diwaniya teaching hospital in Al-Diwaniya city. Samples which were collected by sterile swabs and containers had been cultured on macConkey agar and blood agar, to get pure colonies subculture done on macConkey agar, incubated for overnight at 37°C. The study population was 53.4 % male and 46.5 % female. The specimens were isolated from ear swab (46.2), burn (28%), sputum (14.2%), and urine (11.4%). 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.

Antibiotic Susceptibility Assays: Susceptibility of the selected isolates to four aminoglycoside antibiotics [amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg) and netilmicin (30 µg)]. were tested by using the disk-diffusion method on Mueller Hinton agar according to Clinical and Laboratory Standards Institute (CLSI) guidelines (7) All drugs were obtained from (Bioanalyse company-Turkey). *E. coli* ATCC 25922 served as a control for the disk diffusion test.

Polymerase Chain Reaction Amplification (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')-IIb*, *Aph(3')-IV*, *Ant(4')-IIb* and 16rRNA methylase genes (*armA*, *rmtA*, *rmtD* and *npmA*). Primers were designed from sequences deposited in the GenBank database Table (1). 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 (8). 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 (8). Then, 5µl of each PCR product was analyzed by electrophoresis on 1% (w/v) TAE agarose gel containing 0.1 µl/mL ethidium bromide (9). The amplicon were then visualized on a UV transilluminator and photographed (BioDoc-Analyse; Biometra, Goettingen, Germany).

Table 1: DNA Primer which purchased from Bioneer (Korea) company.

Primer name	Oligosequence (3'-5')		Amplicon	Genbank code
aac (3')-I	F	AGTTTGAGCAAGCGCGTAGT	164bp	AF263520.1
	R	GGGATCGTCACCGTAATCTG		
aac (3')-II	F	CAAACGATGGGTGACGTATG	212bp	AF466526.1
	R	CGTCGAACAGGTAGCACTGA		
aac (6')-I	F	ACTAGGGTTTGCCGAGCTTT	257bp	AF263520.1
	R	AGCAGCGTACTTGAGCAACC		
aac (6')-IIb	F	TCCGTCACCTCCATACATTGC	304bp	DQ174113.1
	R	CGGTACCTTGCTCTCAAAC		
aac (6')-IIb	F	CGCTCGAAGAGGTGAAAGAG	359bp	L06163.1
	R	TGAAACGACCTTGACCTTCC		
Aph3VI	F	CCGAAGACGACATCGGTATG	410bp	DQ315788.1
	R	TGCCTTCTCATAGCAGCGTA		
Ant(4')-IIb	F	TCCTGTACCTGCGAATTGTG	462bp	AY114142.1
	R	CTAGCGCCTCAACGGTATTC		
armA	F	GGTTGTGGCTTCAATCCATTAG	130bp	GQ227508.1
	R	TCGTCGTCTTTAACTTCCCAAT		
rmtA	F	GATTTGCGGGGCTATGTCA	502bp	AB083212.2
	R	GTTTGCTTCCATGCCCTTGC		
rmtD	F	GATCCATTCCGCATTCACGC	552bp	DQ914960.2
	R	AAATATCGCGACGTTTGCCC		

npmA	F	TTGAGGCGTTCTGTGCTGAT	609bp	NG_036511.1
	R	TATGCCGTACCCTTCCAGGA		
gyrA	F	GAGGAAGTGGGAAGCGGTCAA	315bp	NC002516.2
	R	CGGAATCTCGAAGGTACGCA		
gyrB	F	CCGGAGACCTTCAGCAACAT	403bp	FJ652724.1
	R	TGAATTTCTTCGCCAGGCCT		
ParC	F	CTCTCGGAAAAAGGCTGGGT	458bp	AB003428.1
	R	AACAATAGCAGACGGCCCTC		
ParE	F	GAAATGGCGGACGAACAGC	244bp	AY164481.1
	R	GGACAAGGAATTCCAGGCGA		

RESULTS:

A total of 350 clinical samples have been collected, the distribution of clinical samples was explained in table (2). 162 (46.2 %) were collected from ear , 98 (28%) samples were collected from burn while 50 (14.2) and 40 (11.4%). samples were collected from sputum and urine respectively. According to the gender 163 (46.5) samples were collected from female while 187(53.4%) samples were collected from male. Outpatient constituted 208 (59.4 %) and 142 (40.5%) samples from inpatient.

Table 2 : Distribution of 350 clinical samples according to the type, gender and hospitalization.

Type of sample	Gender		Total number	Hospitalization	
	Male	Female		Outpatient	Inpatient
Ear	88	74	162	118	44
Burn	52	46	98	20	78
Sputum	27	23	50	38	12
Urine	22	18	40	32	8
Total	187	163	350	208	142

Only 50 (14.2 %) isolates were belonging to *P. aeruginosa* Table (3) , in which 16.6% of them were isolated from ear swab , 13.2% were isolated from burn while 12% and 10% were isolated from sputum and urine respectively.

Table 3: Incidence of the isolated microorganisms in different clinical sample Sites.

Source of samples	No. of samples	No.(%) of <i>P. aeruginosa</i> Isolates	No.(%) of Gram positive and negative isolates	No.(%) of no growth or contaminated cultures
Ear	162	27(16.6%)	55(33.9%)	80(49.3%)
Burn	98	13(13.2%)	34(34.6%)	51(52%)
Sputum	50	6(12%)	15(30%)	29(58%)
Urine	40	4(10%)	10(25%)	26(65%)
Total	350	50(14.2%)	114(32.5%)	186(53.1%)

The isolated bacteria showed variable results of resistance to aminoglycosides: gentamicin, amikacin, netilmicin and tobramycin. Based on the results from susceptibility testing Table(4) , 32 (64%) of 50 *P. aeruginosa* isolates were found to be resistant to at least one of aminoglycosides, comprising 12 isolates (24%) susceptible and 6 (9.2%) with intermediate susceptibility.

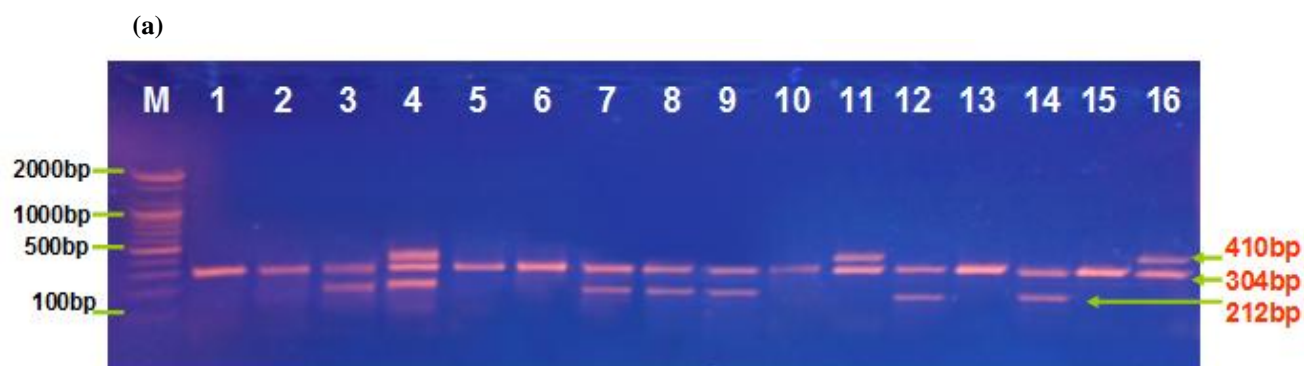
Table 4: Incidence of antibiotic resistance in *P. aeruginosa* isolates (n=50).

Antibiotic	Sensitive	%	Intermediate	%	Resistant	%
Amikacin	30	60	7	14	13	26
Gentamicin	12	24	6	12	32	64
Netilmicin	25	50	10	20	15	30
Tobramycin	17	34	9	18	24	48

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 28 (87.50%) isolate of aminoglycoside resistance isolates. Figure (1 a and b).

Table 5: Distribution of various aminoglycosides-resistance genes and in AACs carried *P. aeruginosa* isolates (n=28).

Occurrence of gene	No.(%) of isolates	Isolate code No.
<i>aac(6')-Ib</i>	28 (87.50%)	1, 2, 3, 5, 6, 8, 9, 10, 12, 14, 15, 16, 17, 19, 22, 24, 26, 27, 28, 30, 32, 33, 39, 42, 43, 47, 49, 50
<i>aac(3)-II</i>	10 (31.25%)	3, 8, 9, 12, 14, 20, 24, 25, 39, 42
<i>aac(3)-I</i>	5 (15.63%)	1, 2, 5, 29, 43
<i>aac(6')-I</i>	7 (21.8%)	3, 5, 8, 9, 12, 19, 30
<i>aac(6')-IIb</i>	3 (8.33%)	3, 8, 28
<i>Aph(3')-VI</i>	8 (25%)	10, 16, 19, 27, 31, 32, 39, 50
<i>Ant(4')-IIb</i>	7 (21.8%)	2, 9, 14, 16, 22, 29, 43
<i>armA</i>	2 (6.25%)	2, 30
<i>rmtA</i>	10 (31.25%)	3, 5, 6, 9, 15, 20, 27, 33, 43, 50
<i>rmtD</i>	6 (18.75%)	9, 14, 15, 19, 33, 39
<i>npmA</i>	4 (14.3%)	5, 6, 10, 15, 16, 17, 20, 22, 27, 28, 29, 39, 49, 50



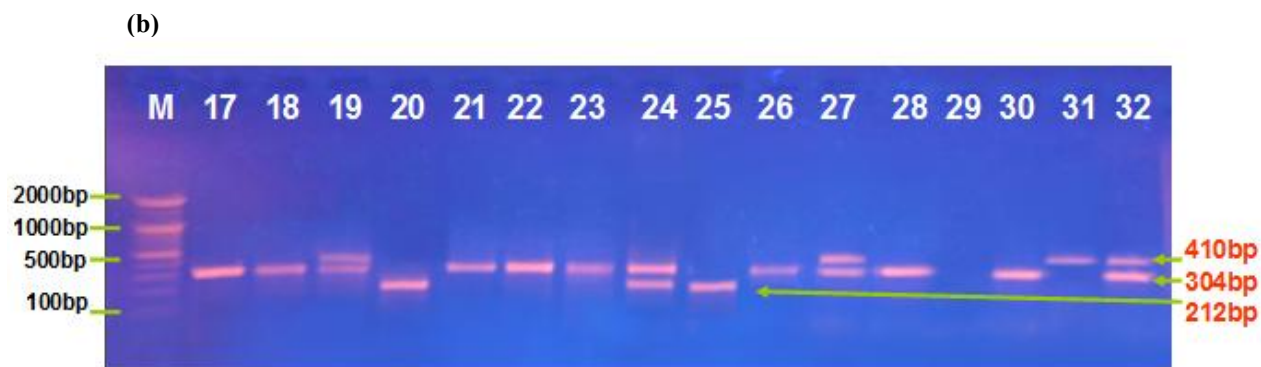


Figure (1a and b): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *Pseudomonas aeruginosa* amplified with *aac(6')-Ib* , *aac(3)-II* , and *Aph(3')-VI* genes primers. The electrophoresis performed at 60 volt for 2 hr. Lane(L), DNA molecular size marker (100-2000bp ladder). Lanes (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 21, 22, 23, 24, 26, 27, 28, 30, 32) of isolates show positive results with *aac(6')-Ib* (304 bp) , Lanes (3, 4, 7, 8, 9, 12, 14, 20, 24, 25) of isolates show positive results with *aac(3)-II* (212bp) , and Lanes (4, 11, 14, 16, 19, 27, 31, 32) of isolates show positive results with *Aph(3')-VI* (410bp).

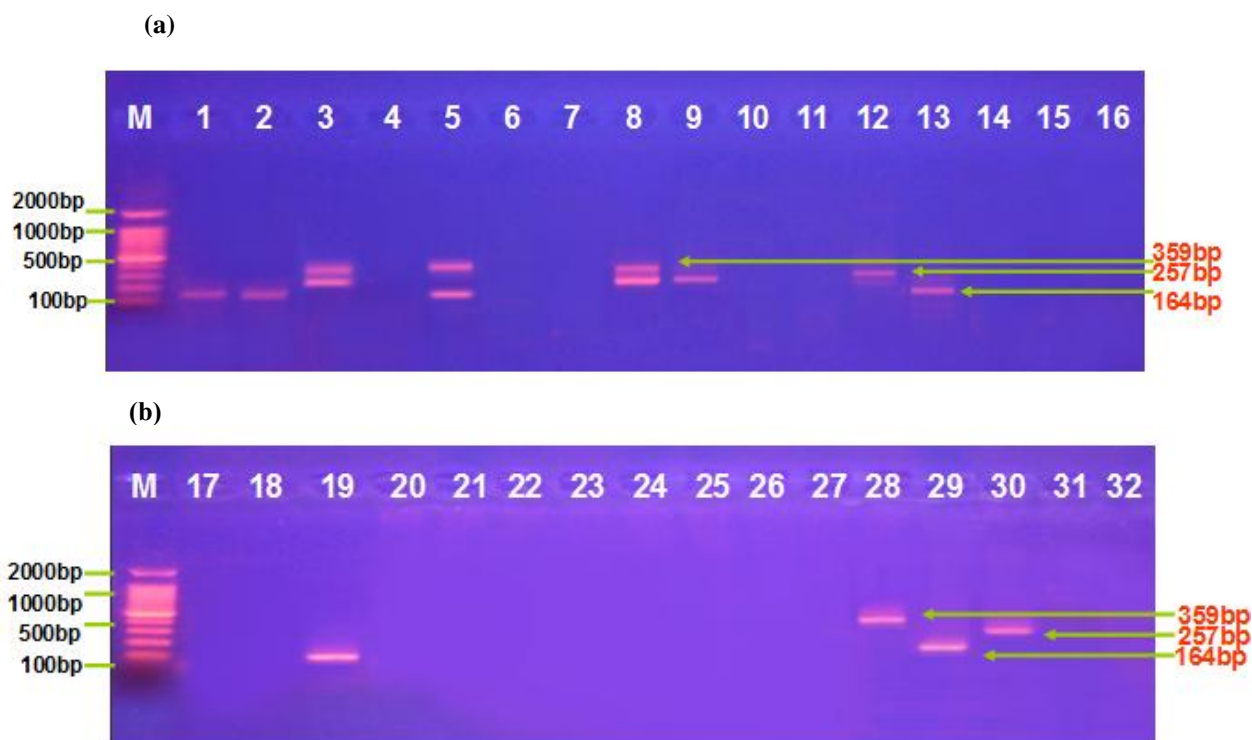


Figure (2a and b): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *Pseudomonas aeruginosa* amplified with *aac(3)-I* , *aac(6')-I* , and *aac(6')-IIb* genes primers. The electrophoresis performed at 60 volt for 2 hr. Lane(L), DNA molecular size marker (100-2000bp ladder). Lanes (1, 2, 5, 13) of isolates show positive results with *aac(3)-I* (164 bp) , Lanes (3, 5, 8, 9, 12, 19, 30) of

isolates show positive results with *aac(6')-I* (257bp) , and Lanes (3 , 8 , 28) of isolates show positive results with *aac(6')-IIb* (359bp).



Figure (3a and b): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *Pseudomonas aeruginosa* amplified with *Ant(4')-IIb* , *armA* ,and *rmtA* genes primers. The electrophoresis performed at 60 volt for 2 hr. Lane(L), DNA molecular size marker (100-2000bp ladder). Lanes (2 , 9 , 13 , 14 , 16 , 22 , 29) of isolates show positive results with *Ant(4')-IIb* (462bp) , Lanes (2 , 30) of isolates show positive results with *armA* (130bp), and Lanes (3 , 5 , 6 , 9 , 11 , 13 , 15 , 20 , 23 , 27) of isolates show positive results with *rmtA* (502bp).



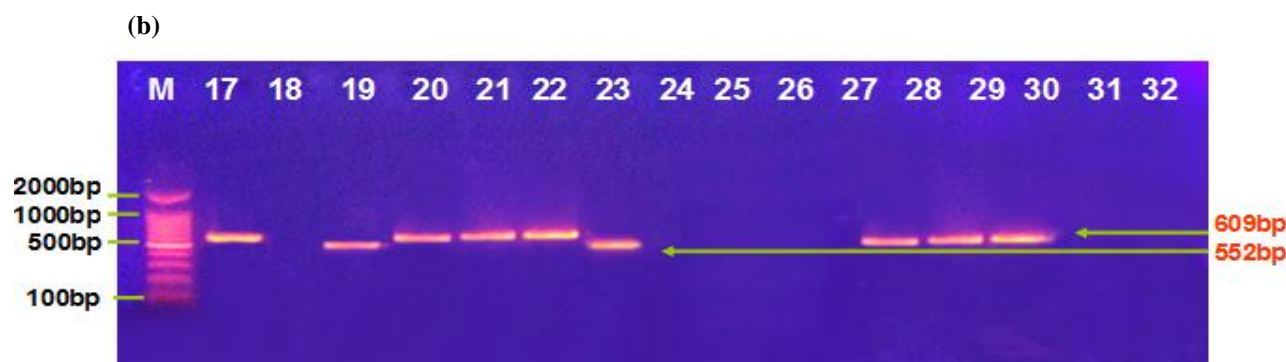


Figure (4a and b): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *Pseudomonas aeruginosa* amplified with *rmtD* and *npmA* genes primers. The electrophoresis performed at 60 volt for 2 hr. Lane(L), DNA molecular size marker (100-2000bp ladder). Lanes (4 , 9 , 14 , 15 , 19 , 23) of isolates show positive results with *rmtD* (552bp) , Lanes (4 , 5 , 6 , 10 , 11 , 15 , 16 , 17 , 20 , 21 , 22 , 27 , 28 , 29) of isolates show positive results with *npmA* (609bp) .

DISCUSSION:

The incidence of *P. aeruginosa* among the examined samples was 50 positive isolates with a percentage of 14.2 %. The result of current study agree with (10 , 11) . Also The result of current study didn't agree with (12 , 13) . The present investigation found that *P. aeruginosa* was most commonly isolated (16.6%) from the ear infection and this consistent with some previous surveillance studies (14 , 15). Previous studies performed burn hospitals in various cities of Iraq (14 , 16), demonstrated high incidence of these isolates. The results of the present study showed that out of the entire burn culture positive, 13.2% exhibited *P. aeruginosa* isolates. The results of the present study showed that 12% isolated from the sputum. This result of the current study comparable with the results of (14) . Out of 40 patients with UTI, 10% patients had an established *P. aeruginosa* etiology. This rate was comparable with many publications studies recorded (17 , 18).

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 (64%) followed by tobramycin (48%) .This acquired resistance is characteristic of high-level resistance to aminoglycosides specifically gentamicin and tobramycin . These rates were comparable with previous reports published (17 , 19),but this result didn't agree with (20).While the present study showed resistance to netilmicin and amikacin among the clinical isolates of *P. aeruginosa* (30%), (26%) respectively, which are consistent with results (21 , 22). 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 (23). 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 (24).

In the present study the aminoglycoside resistance rate in 32 isolates of *P. aeruginosa* . Most of resistant isolates harbored at least one of AACs gene. The *aac(6')-Ib* was the most common detected AAC (87.50%) followed by *aac(3)-II* (31.25). These results are similar to that has been observed in different studies in other countries (19 , 21 , 25), but these results aren't similar to (26). The *aac(3)-I*, *aac(6')-I* , *aac(6')-IIb* were (15.63% , 21.88% , 8.33%) respectively. The result of current study agree with (21 , 27 , 28), but the result didn't agree with (29).

Though *Aph(3')-VI* was present in (25%) of the isolates in the present study , which was high than previous reports (15 , 30), *ant(4')-IIIb* was present in (21.88%) this result was comparable with (15). 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 .

The common 16S rRNA methylase genes were *npmA* (43.75%) and *rmtA* (31.25%) followed by *rmtD* (18.75%) and *armA* (6.25%) this result didn't agree with (21).

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