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Dear Author(s)

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Greetings, with reference (JPT 17) to your article entitled:

MOLECULAR CHARACTERIZATIONS OF AMINOGLYCOSIDE MODIFYING ENZYMES OF PROTEUS MIRABILIS ISOLATED FROM PATIENTS IN *AL-QADISIYAH* GOVERNORATE

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MOLECULAR CHARACTERIZATIONS OF AMINOGLYCOSIDE MODIFYING ENZYMES OF *PROTEUS MIRABILIS* ISOLATED FROM PATIENTS IN AL-QADISIYAH GOVERNORATE

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Abstract:

The aim of this study was evaluate the presence of modified enzymes for aminoglycosides antibiotics *in Proteus mirabilis*. Total of 64 clinical isolates were collected from various sources included urine , middle ear ,wounds ,burns ,high cervical and endometrium cervical ,which was taken from in patients and outpatients in Maternal , and General Teaching Hospital in AL- Diwaniya city during the period from 1/3/2015 till 30/9/2015. Select the minimum inhibitory concentration of a number of antibiotics for aminoglycosides for resistant bacterial isolates which are anti-Gentamycin , Tobramycin , Kanamycin , Streptomycin , Netlimicin the isolates showed their ability to grow in high concentrations ranging from (32-1024)µg / ml , also resisted anti-aminoglycosides and with different rates of Gentamycin , Amikacin ,Tobramycin , Kanamycin , Streptomycin , and Netlimicin , it was in varying proportions (54.68 , 31.25 , 76.56 , 81.25 , 85.93 , 81.25) % , respectively .The prevalence of resistant genes for the anti-aminoglycosides was also studied , the results were also variable for genes *aac(3)-Ia* ,*aac(6')-Ib* ,*ant(4')-IIa* ,*ant(2'')-Ia* and *aph(3'')-Ib* ,in percentages (20 , 40 , 15 , 50 , 50) % ,respectively .

Keywords: Aminoglycoside, Modifing Enzymes, *proteus mirabilis*, PCR, MIC, Aminoglycoside antibiotics.

Introduction

Proteus spp. are widely distributed in nature. They can be found in polluted water, soil and manure, where they play an important role in decomposing organic matter of animal origin [1, 2, 3]. *Proteus* is a genus of Gram-negative bacteria; many of which cause infections in humans [4], and about 90% of these infections are caused by P.mirabilis [5] and is mostly found in people with compromised immune system (6). It is an opportunistic pathogen that is able to cause severe invasive diseases [7] in critically ill and immunocompromised patients [8] . *P.mirabilis* strains can acquire various genes for antibiotic resistance via mobile genetic elements: plasmids, integrons, gene cassettes, and transposons [9]. The most common mechanism of aminoglycoside resistance is enzymatic modification of antibiotic molecule [10]. After modification, the aminoglycoside is not able to bind to the aminoacyl site of 16S rRNA within the bacterial 30S ribosomal subunits and inhibit protein synthesis [11, 12]. Aminoglycoside - modifying enzymes (AME) have been assigned to three classes: aminoglycoside acetyltransferases (AAC), aminoglycoside phosphotransferases (APH), and aminoglycoside nucleotidyltransferases (ANT) [13]. A more specific classification includes the division into subclasses according to the region specificities for aminoglycoside modifications and spectrum of aminoglycoside resistance [14]. To the subclasses with clinical significance among Enterobacteriaceae rods belong following groups: AAC(3)-I, AAC(6')-I, ANT(4')-II, ANT(2")-I, and APH(3")-I.The subclass AAC(3)-I contributes to the resistance to gentamicin, sisomicin, and astromicin, while AAC(6')-I shows activity against amikacin and gentamicin. Enzymes from ANT(4')-II subclass confer resistance to amikacin, isepamicin, and tobramycin, and AME belonging to ANT(2")-I mediate resistance to dibekacin, gentamicin, kanamycin, sisomicin, and tobramycin. The subclass of APH(3")-I enzymes shows activity against streptomycin [10]. The other mechanisms, which also contributes in resistance to aminoglycosides, can coexist in one bacterial cell along with AME [15]. These mechanisms include methylation of 16S rRNA [16], reduced outer membrane permeability [17], efflux pumps and mutations of the ribosomal proteins or 16S rRNA [16].

Materials and methods

Specimens' collection

Different clinical samples such as urine, wound swab, burn swab , high cervical , endometrium cervical and ear swab , ,which was taken from in patients and outpatients in Maternal , and General Teaching Hospital in AL- Diwaniya city during the period from 1/3/2015 till 30/9/2015. The collection process has been conducted according to [18].

Identification of Bacterial Isolates

The isolates were identified according to [19] by using traditional microscopic examination (Gram's stain), colony morphological features on MacConkey agar and blood agar, and standard biochemical tests.

DNA extraction

The total genomic DNA of the *P.mirabilis* was isolated using the DNA extraction and purification kit (Geneaid, USA) according to the manufacturer instructions. DNA preparations were then analyzed by electrophoresis in 1.5% agarose gel.

Polymerase chain reaction

Polymerase chain reaction was used to amplify the entire sequences of the genes studied in this research. The specific primers (Bioneer, Korea) used for the amplification of these genes [20] were shown in (table 1). The PCR mixtures contained: Top DNA polymerase 1U, dNTP (dATP, dCTP, dGTP, dTTP) each: 250µM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, MgCl2 1.5mM, Stabilizer and tracking dye .The cycling parameters of amplification were: Initial denaturation 95°C for 5min, Denaturation 95°C for 30 sec., Annealing (52°C) for 30 sec., Extension 72°C for 1min., Final extension 72°C for 5min.

Agarose gel electrophoresis

The products were separated in 1.5% agarose gel in TBE buffer (pH 8), stained with ethidium bromide, and photographed in ultraviolet light [21].The electrophoresis result noticed by using gel documentation system.

Table (1): Sequences of oligonucleotide primers used in PCR assays for amplification of genes encoding AME

Primer	Sequence	Amplicon
aac(3)-Ia	GGCTCAAGTATGGGCATCAT	389bp
	TCACCGTAATCTGCTTGCAC	
aac(6')-Ib	GCTCTTGGAAGCGGGGACGG	300bp
	TCGCTCGAATGCCTGGCGTG	
ant(4')-IIa	ATCGTCTGCGAGAAGCGTAT	839bp
	TAAAACGCCTATCCGTCACC	
ant(2")-Ia	GACACAACGCAGGTCACATT	500bp
	CGCAAGACCTCAACCTTTTC	
aph(3")-Ib	CTTGGTGATAACGGCAATTCC	548bp
	CCAATCGCAGATAGAAGGCAA	

Antimicrobial susceptibility test

Susceptibility to antimicrobial agents was determined by using the disk diffusion method [22]. The following antimicrobial agents (obtained from Bioanalyse Turkyi ,Oxoid England) were used: gentamycin (GM), amikacin (AK), Tobramycin (TOB), Kanamycin (K), Netilmicin (NET) and Streptomycin (S).

Susceptibility to antimicrobial agents was determined by using the minimum inhibitory concentrations (MICs) [23]. The following antimicrobial agents (obtained from

Kocaeli Turkyi, Hamburg Germany) were used: gentamycin (GM), Tobramycin (TOB), Kanamycin (K), Netilmicin (NET) and Streptomycin (S).

The inoculums were prepared by growing the various *Proteus mirabilis* on separate agar plates and colonies from the plate were transferred with inoculating loop into 3 ml of normal saline in a test tube. The density of these suspensions was adjusted to 0.5 McFarland standards. The surface of Muller-Hinton agar (Himedia India) plate was evenly inoculated with the organisms using a sterile swab. The swab was dipped into the suspension and pressed against the side of the test tube to remove excess fluid. The wet swab was then used to inoculate the Muller-Hinton agar by evenly streaking across the surface. The antibiotic discs were applied to the surface of the inoculated agar and the plates were incubated overnight at 37ºC. The diameter of zone of growth - inhibition observed was measured and compared to the chart provided by National Committee for Clinical Laboratory Standards (NCCLS).

Results and Discussion

Isolation and identification

Sixty four isolates were identified as *P. mirabilis* consisted of ٣٢ isolates (12.8%)obtained from urine , 17 isolates (9.18%) obtained from middle ear, 3 isolates (7.5%) obtained from wounds , 4 isolates (4.7%) obtained from burns and 8 isolates (8.88%) obtained from high cervical and endometrium cervical (table 2).

Table (2): Distribution of *P.mirabilis* among various clinical sources.

Type of samples	No. of samples (%)
Urine	32 (12.8)
Middle ear	17 (9.18)
Wounds	3 (7.5)
Burns	4 (4.7)
High cervical and endometrium cervical	8 (8.88)
Total	64 (9.84)

We analyzed resistance to 6 antibiotics aminoglycosides all isolates showed micro-biological resistance (table 3), Gentmycin about (54.68%) this result agrees with (24) about (50%) but not agrees with [25] about (33%), also Amikacin (31.25%) this result agrees with [26] about (38.4%) but this current result not agrees with [27,24] about (1.6,5)% , respectively. Tobramycin (76.56%) this result agrees with [28] about (81%) but this current result not agrees with [29] about (33.3%). also antibiotics Kanamycin, Streptomycin and Netlimicin about 81.25, 85.93, 81.25%, respectively.

Table (3): Percentage (%) of antimicrobial resistance and sensitivity of *P.mirabilis* .

Antibiotics	Resistance isolates		Sensitive isolates	
	No.	(%)	No.	(%)
Gentamycin	35	54.68	29	45.31
Amikacin	20	31.25	44	68.75
Tobramycin	49	76.56	15	23.43
Kanamycin	52	81.25	12	18.75
Streptomycin	55	85.93	9	14.06
Netlimicin	52	81.25	12	18.75

We analyzed resistance to five antibiotics for 20 isolates under study were subjected to test the minimum inhibitory concentrations (MICs) in (table 4) , value of MIC to Gentamycin were (32-512)µg/ml this result agrees with [30] were MIC value (8 ≤ 512-)µg/ml , but this current result not agrees with [31] were MIC value (0.5-256) µg/ml , also Tobramycin were MIC (32-512) µg/ml which is a break point (8 ≤) µg/ml , this result agrees with (32) were MIC value for Tobramycin (16-512) µg/ml but this current result not agrees with [31] were MIC value (0.5-256) µg/ml , also study [30] were MIC value for Tobramycin (4-1024) µg/ml , Kanamycin in the current study were MIC vaule (32-1024) µg/ml which is a break point (16 ≤) µg/ml , this result approach with [31] were MIC value (2-1024) µg/ml , while Streptomycin and Netilmicin in the current study were MIC value (128-1024)µg/ml which is a break point (32 ≤ , 16 ≤) µg/ml , respectively.

Table (4): Minimum inhibitory concentrations (MIC) for ant *P. mirabilis* isolates.

Antibiotic / No.S.	Break point	NET 16≤ g / mlµ	S 32≤ g / mlµ	K 16≤ g / mlµ	TOB 8≤ g / mlµ	CN 8≤ g / mlµ
1		1024	1024	128	512	256
2		1024	1024	512	512	256
3		1024	1024	64	64	256
4		128	512	1024	64	256
5		512	512	1024	32	512
6		256	512	256	256	512
7		256	256	256	32	128
8		256	128	256	64	256
9		256	512	512	512	64
10		128	1024	512	512	64
11		512	1024	32	128	512
12		1024	1024	512	256	512
13		1024	512	1024	128	64
14		1024	1024	1024	64	64
15		256	256	128	128	64
16		128	256	32	512	128
17		256	1024	32	512	256
18		512	128	128	32	256
19		256	512	512	256	128
20		256	256	64	256	128

Aminoglycosides (Amgs) are highly potent, broad spectrum antibiotics with many desirable properties for the treatment of human infections. Among resistance mechanisms to Amgs inactivation by aminoglycoside-modifying enzymes (AMEs) is the most important both in terms of level and frequency of resistance conferred to the bacterium [33].There are three family of AMEs including aminoglycoside acetyltransferases (AACs), phosphotransferases (APHs), and nucleotidyltransferases (ANTs). Each group of enzymes consists of different isozymic forms that differ in substrate regiospecificity for their reactions [33]. Amgs modification has emerged in all clinically relevant bacteria of both Gram-positive (*Staphylococcus* spp., *Enterococcus* spp.) and Gram negative, including *Proteus* spp. strains [33, 34, 25, 35]. These enzymes are often plasmid-encoded but may also be associated with transposable elements facilitating the rapid dissemination of the genes in various bacterial populations [33, 36]. In this study we present occurrence

of modified enzymes for aminoglycosides antibiotics in clinical strains of P*roteus mirabilis*. The genes encoding AME were detected in 20 *P.mirabilis* isolates which represents five genes (Table 5). The results of the current study showed that the presence of the gene *aac(3)-Ia* in *P.mirabilis* about (20%) (Fig.1), while a study showed [37] presence the gene *aac(3)-Ia* about (1.51%) .The presence of *aac(6')-Ib* about (40%) (Fig.2) this result agrees with [38] about (36.5%), also agrees with [39] about (37.28%), but the result of the current not agree with (40) about (71.43%). While The presence of ant*(4')-IIa* about (15%) (Fig.3) this result approach with [37] the presence of *ant(4')-IIa* gene was not detected in any of tested isolates.

Also the presence of the gene *ant(2")-Ia* about (50%) (Fig.4), while a study showed [37] about (80.30%), but not agree with (41) about (36%). Finally, the study showed that the presence of the gene *aph(3")-Ib* about (50%) (Fig.5), this result not agree with [37] about (21.21%).

Table (5): Prevalence of genes encoding AME in *p.mirabilis* isolates

Genes	No. *P.mirabilis* isolates	(%)
aac(3)-Ia	4	20
aac(6')-Ib	8	40
ant(4')-IIa	3	15
ant(2")-Ia	10	50
aph(3")-Ib	10	50

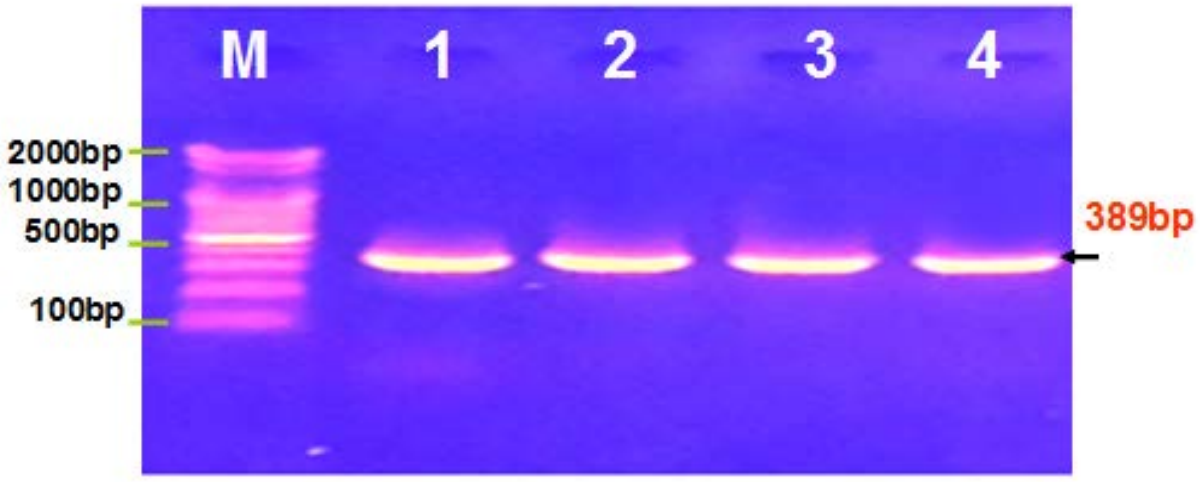

Fig.1. Demonstration of *aac(3)-Ia* gene resistant for antibiotic aminoglycosides with PCR in agarose gel. M: Marker ladder 100-2000bp, Lanes 1-4 positive numbers for the gene *aac(3)-Ia* of clinical *Proteus mirabilis* (80mA and 100V) for one hour.

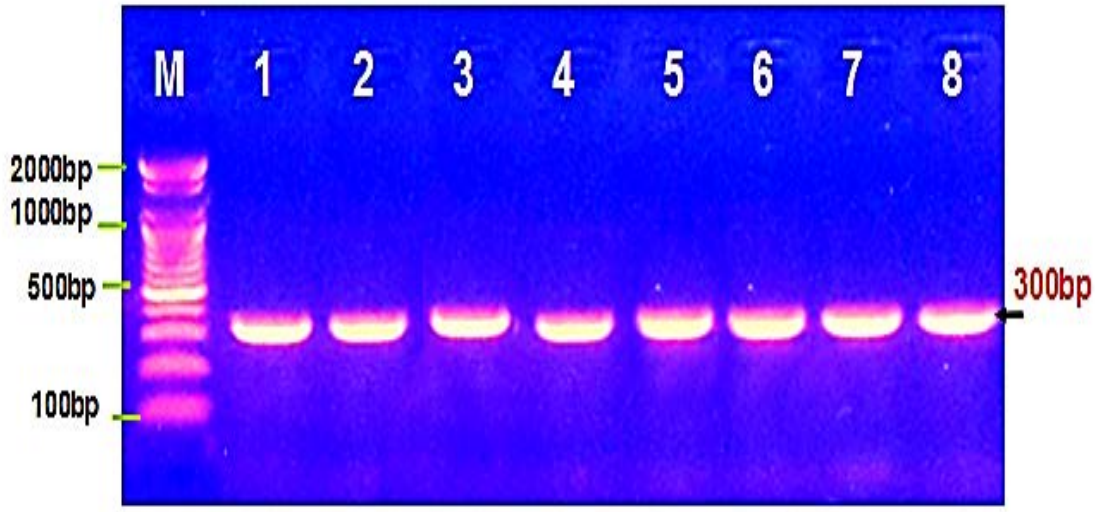

Fig.2. Demonstration of *aac(6')-Ib* gene resistant for antibiotic aminoglycosides with PCR in agarose gel. M: Marker ladder 100-2000bp, Lanes 1-8 positive numbers for the gene *aac(6')-Ib* of clinical *Proteus mirabilis* (80mA and 100V) for one hour.

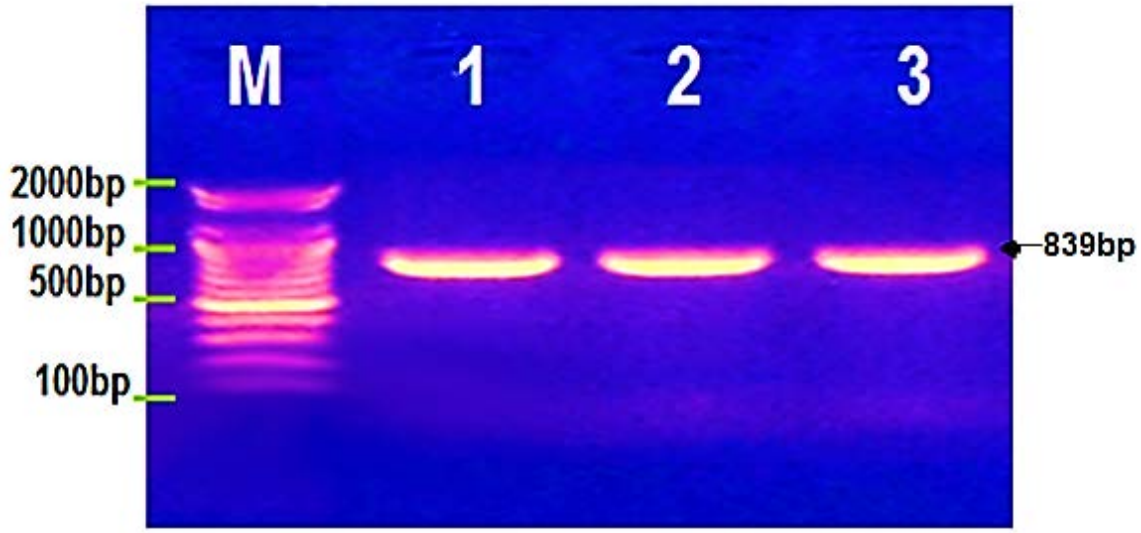

Fig.3. Demonstration of *ant(4')-IIa* gene resistant for antibiotic aminoglycosides with PCR in agarose gel. M: Marker ladder 100-2000bp, Lanes 1-3 positive numbers for the gene *ant(4')-IIa* of clinical *Proteus mirabilis* (80mA and 100V) for one hour.

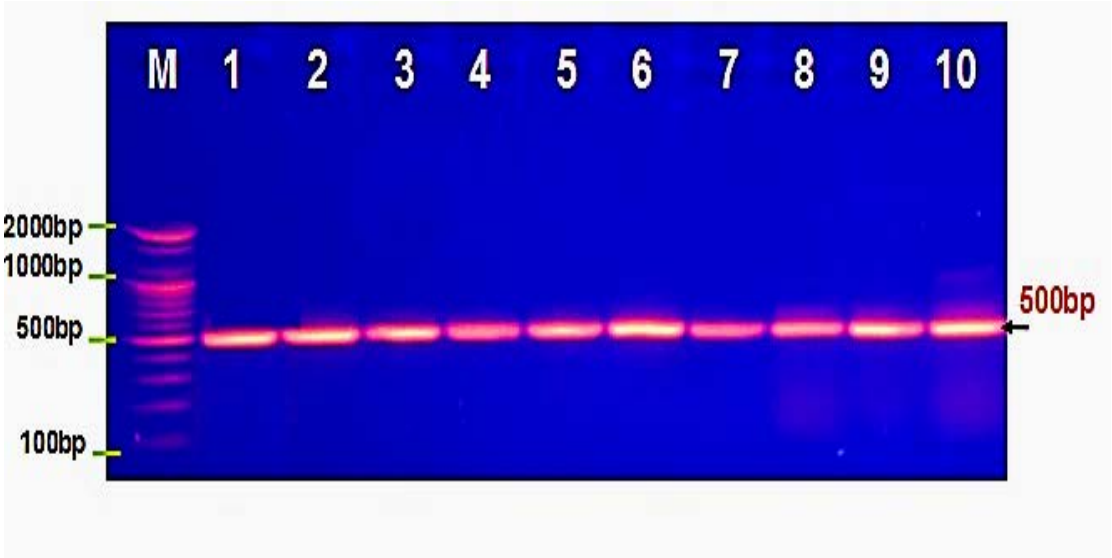

Fig.4. Demonstration of *ant(2")-Ia* gene resistant for antibiotic aminoglycosides with PCR in agarose gel. M:Marker ladder 100-2000bp, Lanes 1-10 positive numbers for the gene *ant(2")-Ia* of clinical *Proteus mirabilis* (80mA and 100V) for one hour .

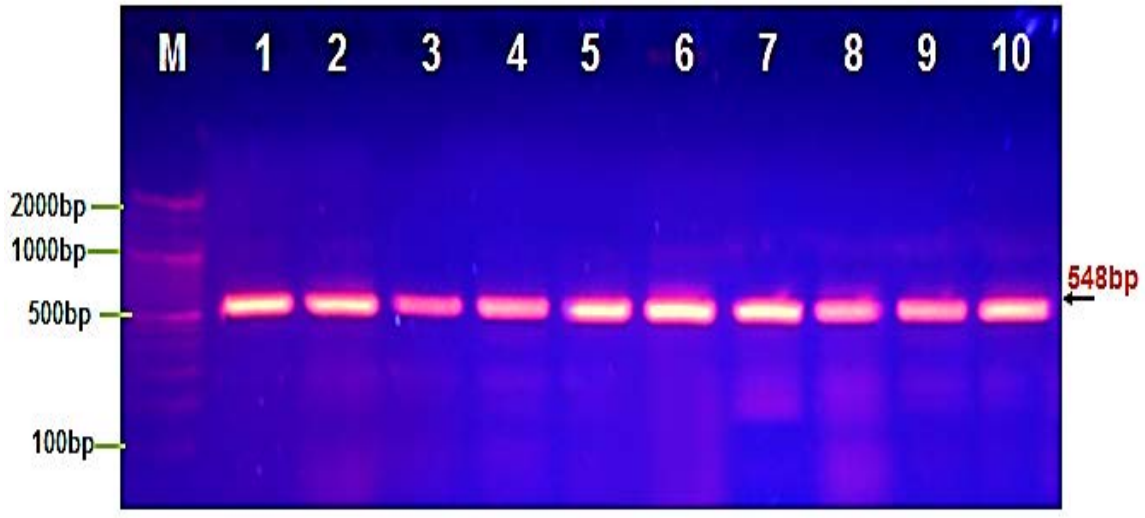

Fig.5. Demonstration of *aph(3")-Ib* gene resistant for antibiotic aminoglycosides with PCR in agarose gel. M: Marker ladder 100-2000bp, Lanes 1-10 positive numbers for the gene *aph(3")-Ib* of clinical *Proteus mirabilis* (80mA and 100V) for one hour

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