Plant Archives Volume 20 No. 2, 2020 pp. 4795-4800 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

MOLECULAR STUDY OF *PSEUDOMONAS AERUGINOSA* ISOLATED FROM EAR INFECTION OF GOAT IN AL- DIWANIYA CITY, IRAQ BY PCR BASED ON OPRL AND OPRI GENES WITH DETERMINATION OF THE TWO AMINOGLYCOSIDE RESISTANCE GENES

Moshtaq Talip Hussein[1], Ali Mohammed Ghazi[2*] and Ishtar Adnan Mohammed[2]

[1]Dentistry College, University of Al-Qadisiyah, Iraq.
[2*]Veterinary College, University of Al-Qadisiyah, Iraq.

Abstract

Fifty samples of infected ears canals of goat are collected by sterile cotton swab. All samples was cultured on culture media performed by streaking method to isolated the *P. aerogenosa*. The pure colonies are identified by morphological, cultural and biochemical techniques followed by detection molecularly by a oprL and oprI genes. The results of aminoglycoside resistance was detected by using disc diffusion method. Amikacin was the most powerful one since the percentages of resistance were 50% then Tobramycin in percentage 70% while all isolates showed 100% resistant to Gentamicin and Kanamycin. Isolates of *Pseudomonase aeruginosa* were used to detect the antimicrobial resistance to aminoglycoside by mexX and mexY genes through the use of PCR technique. Seven isolates (70%) showed positive results to mex X and mex Y respectively. This may be indicated to the spread of those kinds of resistant.

Key words: *Pseudomonas aeruginosa*, Antimicrobial resistance, MexY,, MexX. Aminoglycoside, Efflux system.

Introduction

The main virulent agent was *Pseudomonas aeruginosa* in contaminations (Van Eldere, 2003; Xia and Tang, 2016). The common vital issue in an inhalation of *P. aeruginosa* is the habitually watched several-drug resistance mechanism moreover, *P. aeruginosa* can likewise get imperviousness to different antimicrobial specialists, for example, aminoglycosides, fluoroquinolones and β-lactams; are a vital part of anti-pseudomonal chemotherapy and they display collaboration with β-lactams (Burdon *et al*., 1967). A common biological problem with the extermination of *P. aeruginosa* is the mechanism of resistance of many drugs that are routinely monitored. Moreover, *P. aeruginosa* can also be impervious to various antimicrobial specialists, for example, aminoglycosides, fluoroquinolones and β-lactams; they are a vital part of an anti-toxin chemotherapy and they show collaboration with β-lactams.

P. aeruginosa is bacteria of the more significant pathogen which give rise to a rising average of infection and death in patients in hospital with weakened immune systems (Burdon and Whitby, 1967; Cornelis *et al*., 1989). Generally, antibiotics were used to treat infections *by Pseudomonas* spp., however, unluckily, in Hospital, the treatment of the infectious patients with these bacteria are becoming most difficult due to the increase of antibiotic resistant strains numbers. Recently, the most problems infections in hospitals are caused by this bacterium and caused high mortality rates from 18-61% (Grundmann *et al*., 1993).

The interaction between *P. aeruginosa* and its environment was performed through the bacterium protein of outer membrane (Hancock *et al*., 1990). The origin of antibiotics resistance of *P. aeruginosa* in minimum for several types of antibiotics, certain protein of external membrane presence which was participate in efflux system of transportation and due to permeabilization of affect cell (Masuda *et al*., 1995).

Emergence of antibiotics resistance is taken a high concern in the health of human. *One of the most important pathogen in hospital is Pseudomonas*

***Author for correspondence :** E-mail: ali.ghazi@qu.edu.iq

aeruginosa and its infections (Akama *et al*., 2004). The antibiotics expulsion is main mechanism of antibiotic resistant in *this type of bacteria* and the systems of several antibiotics resistant efflux consider one of the resistant-nodulation-branch (Schaible and Taylor, 2012). Significantly, MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY, MexJK and MexVW participate in the more resistance for antibiotics (Poonsuk *et al*., 2014). The expressed mechanism of the MexAB-OprM efflux pump is responsible for resistance of *P. aeruginosa* to multiple antibiotics (Yoneyama *et al*., 2000).

P. aeruginosa has many systems of several antibiotics efflux (MexAB-OprM, MexCD-OprJ, MexEF-OprN andd MexXY-OprM) and should be noted as most important determinants of several antibiotics resistant in many bacteria (Poole, 2004). This bacteria consider as member of the more widespread nosocomial bacteria have relationship with raising death average and antibiotic price (Farhat *et al*., 2009).

Materials and Methods

Samples collection

Fifty samples of infected ear canals of goats are collected by sterile cotton swab and put them in peptone water and incubation for 37ºC at period 24 hrs.

Isolation and Identification of Bacteria

All samples was cultured on culture media performed by streaking method on nutrient agar, blood and MacConkey agar then incubation at 37ºC for 24-48 hrs. The pure colonies are identified by morphological, cultural and biochemical techniques (Koneman *et al*., 1983; Cater and Chengappa, 1993; Quinn *et al*., 1994).

Antibiotic susceptibility test

Susceptibility of Bacteria to antimicrobial drug was done by using of the disc diffusion test. This test was done by spreading the bacterial colonies on the Muller Hinton medium surface, then antimicrobial discs placed on medium surface. These dishes were incubated at 37ºC for 24 hrs. for the purpose of assessing the bacterial resistance to these antibiotics. The zones of inhibitory were measured in mm and compared with (CLSI) (CLSI, 2009). Aminoglycosides used in this study were: Amikacin, Gentamicin, Tobramycin and Kanamycin.

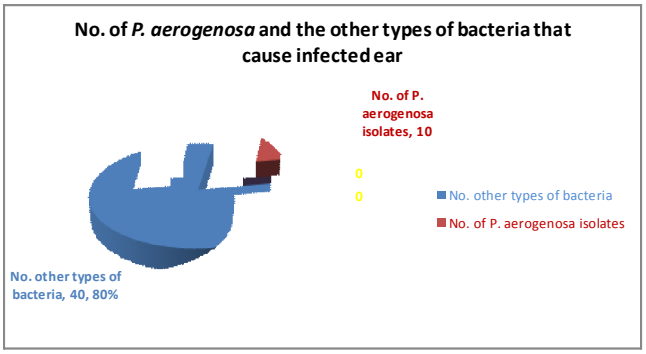

Fig. 1: The numbers and percentages of *P. aerogenosa* and other types of bacteria that cause the ear infections.

DNA extraction

Lessening the contaminations then the probability of untruthful-positive result, all procedures of DNA extraction were accomplished in a place physically secluded both from that utilized to performed the amplification of nucleic acid and from the post-PCR place. Extraction of gDNA of Bacteria were done from all strains phenotypically and biochemically were tested by a boiling method. For this way and depending on company instructions (Bioneer, Korea), suspension of bacteria were performed by choice 3-6 colonies were from Petri dish and mix to 0.25 ml water free from DNase / RNase in 1.5 ml eppendorf tube (~1-2×10^9 cells/ml). The suspension was putted in water bath until boiling for ten minutes to the cell lyse, after that centrifuged for ten minutes at 10000g at 4°C. Eventually, another tube was used to transfer the supernatant in clean condition and utilized as template. Storage of Extracted DNA at -20°C until PCR amplification (Lim *et al*., 2012; Hamzah and Hasso, 2019).

Molecular detection

- **Primer selection:**

In this study, The primers was used are shown in table 1 and 2. Genus of *Pseudomonas* was detected by PCR amplification of I lipoprotein (*OprI*) and *P. aeruginosa* species detection was performed depending on L lipoprotein (*OprL*). all these tests were done on the isolates.

Korea (Bioneer company) was responsible for design The primers then (PCR mix master combine) was done by treat with mixture (AccuPower® multiplex PCR mixture kit. Bioneer).

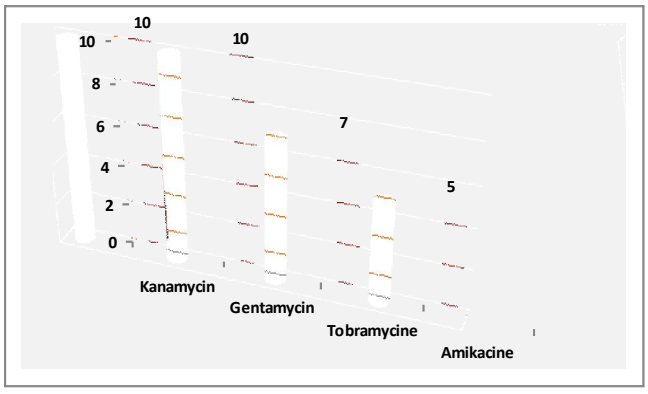

Fig. 2: Number of isolates which show resistance to different antibiotic types that used in the susceptibility test of this study.

• Detection of the opr L and opr I:

To diminish contaminations, all mixtures of reaction were formed in a separated PCR place from the other was used to genetic material extraction as well as amplification and from the place of post-PCR. adapted polymerase chain reaction micro centrifuge tubes was used to complete PCR depending on the thermocycler. as following, 25 µl of the Reaction mixtures were formed by: eleven µl DNase / RNase-free water, 8 µl 2× PCR Master Mix (1.5 mM mgcl2, Denmark), 0.5 µl of each set of primers (*OprL* or *OprI*) and 5 µl of template. The following parameters of optimized thermal cycling in a thermocycler (Senso- Quest Labcycler, Germany) used to subjects. The reaction mixtures : 94°C for five minutes, then thirty cycles of 94°C for 1 minute, 55°C for one min, 72°C for one minute and a terminal extension at 72°C for ten minutes.

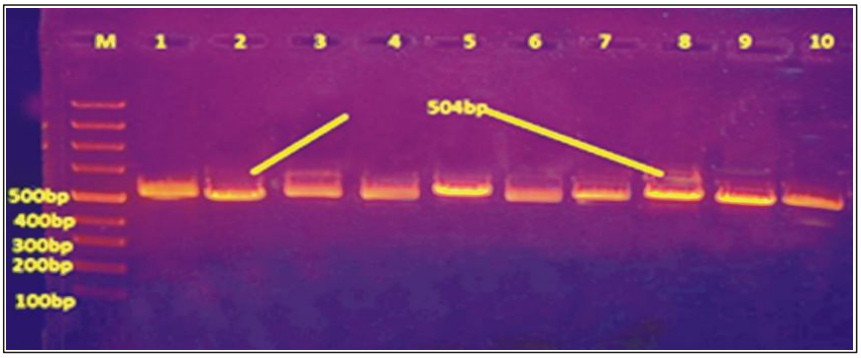

Fig. 3: Gel electrophoresis (agarose 1%, 7 V/cm for 90 mins) of Polymerase chain reaction products of OprL (504 bp amplicons) in- bacterial isolate.. lane M (DNA ladder) 100 base pair molecular markers. bands with OprL genes obtained from *P. aeruginosa* isolates, all isolates were positive results.

Table 1: Study primers used in PCR.

Primer	5'-sequence-3'	Product length (bp)	Reference
OprI-F	ATGAACAACGTTCTGAAATTCTCTGCT	249	(De Vos *et al*., 1997)
OprI-R	CTTGCGGCTGGCTTTTTCCAG		
OprL-F	ATGGAAATGCTGAAATTCGGC	504	(De Vos *et al*., 1997)
OprL-R	CTTCTTCAGCTCGACGCGACG		

Table 2: Primer sequence and amplicon sizes.

Target genes	Primer sequences	Product sizes (bp)	References
mexX-F	TGAAGGCGG CCCTGG ACATCA GC	326	**(Dumas *et al*., 2006)**
mexX-R	GATCTGCTCGACGCGGGT CAG CG		
mexY-F	CCGCTACAACGGCTATCCCT	250	**(Xavier *et al*., 2010)**
mexY-R	AGCGGGATCGACCAGCTTTC		

Table 3: Polymerase chain reaction conditions for the amplification genes.

The gene	Initial denaturation	Cycles no.	Denat-uration	Annea-ling	Elong-ation	Final extension
MexX	95ºC / 5 min	35	95ºC / 1 min	62ºC/ 1 min	72ºC/ 1 min	7min/ 72ºC
MexY	95ºC / 5 min	30	94ºC / 30 sec	59ºC/ 30 sec	72ºC/ 1 min	7min/ 72ºC

Table 4: Numbers and percentages of isolates which show resistance to different antibiotic types that used in the susceptibility test of this study with.

No.	Types of antibiotics	Kanamycin	Gentamicin	Tobramycin	Amikacin
1	No. of isolates	10 **(100%)**	10 **(100%)**	7 **(70%)**	5 **(50%)**

• Detection of amplicons *ope L* and *opr I*:

After amplification, ten µl of aliquots are segregate from each reactive admixture then tested through technique of electrophoresis (80 V, 45 min) in 1% agarose gels in TBE buffer (40 mM Tris, 20 mM boric acid, 1 mM EDTA, pH 8.3). Gel was examined through UV luminescence by using analysis system of the images (UVitec, Cambridge, United Kingdom) then the image was saved. When a bands were found in the accurate expecting sizes of *OprI*, the results of samples were refer to positive for *Pseudomonas* genus; and if the bands were placed at the accurate expecting sizes for *OprL* , the sample results were refer to positive for species of *P. aeruginosa*.

• Detection of mexXY-OprM genes:

Polymerase chain reaction was utilized the mex-X (sizes of amplification 326 bp) mex-Y (sizes of amplification 250 bp). These primers are recorded in (Table 2). The mixture of a reaction was set up as per the method recommended by a production companies (KAPA, south Africa).

DNA templates were set up through heating five fresh colonies in 25 µl distillated water for ten mins. The components of PCR mixture : five µl templates, 12.5 µl of Go Taq® Green Master Mix (2×), 1.5 µl from forward and reverse primers (terminal concentration 10pmol) of both genes and the final volumes were completed to

25µl of nuclease free water using 4.5 µl for each genes. The following conditions were used in PCR and listed in (Table 3). gel electrophoresis was used to examination of the products of PCR and seen beneath ultraviolet light. This is recommended by Sambrook and Russell (Sambrook and Russell, 2001).

Results and Discussions

Bacteria isolation

This type of bacteria was deem as the main causes of 10-15% of the hospital infections in world. Often the treatment of these infections are difficult because the normal resistant species, in addition to their notable capacity to get the resistance strategies to several types of antibacterial drugs (Procop, 2007). Ten isolates of bacteria are obtained from infected ears clinically. Fig. 1 shows the numbers and percentages of those isolates. All isolates were identified according to the morphology of the colonies and biochemically.

The largely usage of Aminoglycosides are in clinic, chiefly in therapy of highly contagious infection that result from Gram-negative micro-organism (Spilker *et al*., 2004; Tijet *et al*., 2011; Hasso and Al-Janabi, 2019). MIC values were got from the results of other works showed that isolates appeared very high resistant percentages to aminoglycoside antibiotics. The cause of the high percentages of antimicrobial resistance related to several factors. These factors can be transferred of resistant gene from one to another and mutation of gene to high variant of resistance by the bad uses of antibiotics (Turnidge, 2003).

This study show that Amikacin was the most powerful one since the percentages of resistance were 50% then Tobramycine in percentage 70% while all

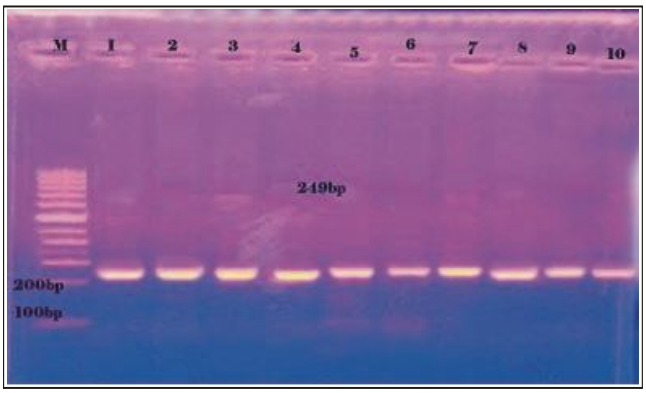

Fig. 4: Gel electrophoresis (agarose 1%, 7 V/cm for 90 mins) of Polymerase chain reaction products of OprI (249 bp amplicons) in- bacterial isolate.. lane M (DNA ladder) 100 base pair molecular markers. Bands with OprI genes obtained from *P. aeruginosa* isolates, all isolates were positive results.

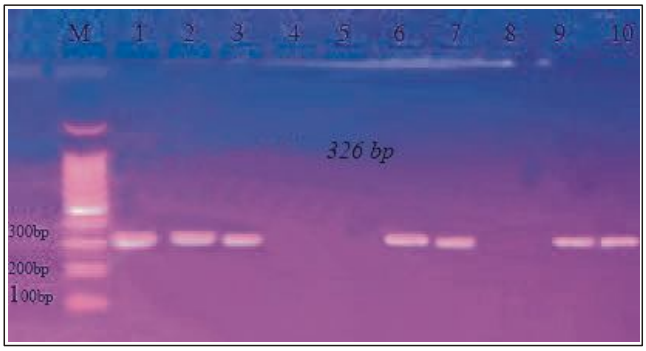

Fig. 5: Gel electrophoresis (agarose 1%, 7 V/cm for 90 mins) of Polymerase chain reaction products of mex X (326 bp amplicons) in- bacterial isolate. lane M (DNA ladder) 100 base pair molecular markers. bands with mex X genes obtained from *P. aeruginosa* isolates, all isolates were positive results., positive lanes are 1, 2, 3, 6, 9 and 10 whilst negative lane are 4, 5 and 8.

isolates showed 100% resistant to Gentamicin and Kanamycin. Table 4 and fig. 2, shows a comparison between the percentages of resistant to aminoglycoside.

Ten samples were detected by molecular techniques. All isolates were showed positive for *P. aeruginosa* species. Polymerase chain reaction test used both primers pairs and result DNA product of the prophesy size. (Fig. 3 and 4). All *P. aeruginosa* isolates showed possession of the *OprI* and *OprL amplicon* genes. AlJabiri *et al*., (2015) used rpsL genes to detect the *P. aeruginosa* and recorded as positive results.

The consequence of the present examination similar to a consequence of Kim *et al*., (2008) that recognized 55 isolates of *P. aeruginosa*, 31% of them are not show sensitive to Amikacin whilst Haldorsen, (2011) recorded averages of insensitive to Amikacin were 30% that agreement with this research.The work of Al-kadmy,

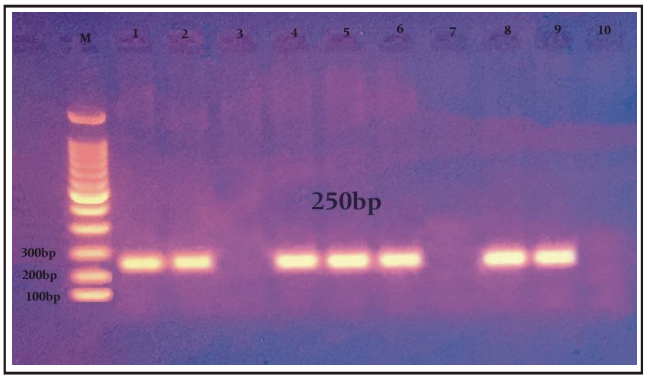

Fig. 6: Gel electrophoresis (agarose 1%, 7 V/cm for 90 mins) of Polymerase chain reaction products of mex Y (250 bp amplicons) in- bacterial isolate.. lane M (DNA ladder) 100 base pair molecular markers. bands with mex Y genes obtained from *P. aeruginosa* isolates , positive lanes are 1,2,4,5 6,8 and 9 whilst negative lane are 3, 7 and 10.

(2012) was recorded (47%) that was agree with the present research. Dubois *et al*., (2008) found the average of resistance for Gentamicin was 55.8% so that this average was not equal to result of a present test.

Otherwise, Ozer *et al*., (2012) view the average of insensitive to kanamycin 100%, whilst, Alkadmy, (2012) recorded the average of proportion of the antibiotics were 92.8%. All results will refer to several resistant methods divided to medication obstruction because of production of the other enzymes coded by plasmids or chromosomes or disorder in utilization of medications that caused from defect in permeability resistance as well as change in the objective for the drug activity with the new discovery of methylation method (Poole, 2005; Giedraitiene *et al*., 2011). Ten isolates were used to detection of two AG resistant mechanisms. MexX genes were detected in 7 (70%) isolates and For mexY gene it was detected in 7 (70%) isolates. (Fig. 4 and 5).

Cabot *et al*., (2011) appeared an expansion average of mexY genes without high expression was 81.5%, the result of them was not agreement with present work, whilst Ozer *et al.,* (2012) explain the spread average of mexX was 4% and that dissent with the present work.

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