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Analysis of antibiotic resistance gene expression in *pseudomonas aeruginosa* by quantitative real-time-PCR

Suhad Hassan Friyah and Marrib N. Rasheed

Institute of Genetic Engineering and Biotechnology for Post Graduate Studies, University of Baghdad, **Iraq**

***Correspondence:** suhadhassan1966@gmail.com **Accepted:** 31 Jan.2019 **Published online:** 21Feb. 2019

Pseudomonas aeruginosa is one of the most common nosocomial pathogens with high mortality rates. Organisms such as *Pseudomonas aeruginosa* have the ability to develop high level MDR (Multi drug resistance). The *MexX* gene is one of the largest gentamicin resistant efflux pumps. Aim of study For better understanding of the antibiotic resistance mechanism in *P. aeruginosa,*it was conducted to determine the expression of the gene encoded these efflux pumps in 54 isolates of *P. aeruginosa* isolated from patients admitted to various hospitals in Baghdad/ AL-Kharkh side using qRT-PCR, were selected based on their gentamicin susceptibility pattern. Antibiotic disk diffusion method was performed for gentamicin. Finally, qRT-PCR was used for determining the efflux pump genes expression related with gentamicin resistance. Among seven isolation sources , the burns showed greatest resistance (100%, n = 14) while the urine iolates was lowest (50%, n = 2) as shown table (II). Most of isolates (88.8%, n = 48) exhibited efflux pump *MexX* gene but different expression was observed in different isolates.. In addition, there was high level of *MexX* gene expression in burn and the lowest was to hospital equipment isolates. Identifying the bacterial resistance mechanisms is very complicated. Although efflux pump *MexX* gene plays an important role in antibiotic resistance *i*n *P. aeruginosa*, because of acting the efflux pumps on antibiotics in a non-specific manner. It is elusive to describe an antibiotic resistance based on the presence or absence of an efflux pump genes.

Keywords: efflex pumps, gentamicin, *MexX gene Pseudomonas aeruginosa andRT-PCR.*

INTRODUCTION

Pseudomonas aeruginosa is capable of growing in a wide variety of niches with a preference for moist environments. In addition, *P. aeruginosa* is one of the three most abundant bacterial species causing nosocomial infections in intensive care units. (R.Spencer, 1996). *P. aeruginosa* has emerged as one of the most problematic nosocomial pathogens, it is considered an opportunistic pathogen that causes infection in immune depressed subjects (Brooks et al.,2007) It is the leading cause of wound infections, urinary tract, surgical wound and ear infection (Todar, K. (2008). *P. aeruginosa* infections are nosocomial in nature, hospital reservoirs of growth are many and include respiratory equipment, solutions, medicines, disinfectants, sinks, mops, food mixers and vegetables .(Trautmann et al., 2005) and (Poole,2004).

Pseudomonas aeruginosa owes its intrinsic and acquired resistance to antimicrobial agents, biocides, organic solvents and heavy metals mostly to the expression of chromosomally encoded efflux pumps belonging to the so-called resistance-nodulation-cell division (RND) family of multidrug transporters (Relman et al.,1992) A rapid and accurate system for the identification of

Pseudomonas is important to isolate patients and prevent further spreading of the diseases.
For the genetic identification and characterization of *pseudomonas species* via PCR-based methods, various targets have been reported, such as *rpsl* gene (Moore et al.,1996)

The *16S* ribosomal RNA (rRNA) gene is most commonly used, but it is not feasible to develop highly specific primer using this gene because of the high similarities of the *16S*rRNA gene sequences (Yamamoto et al.,2000) and (Lavenir et al.,2007)

Real-time PCR assayas a highly sensitive and specific assay when tested with *P. aeruginosa* strains and species of pseudomonads closely related to *P. aeruginosa (*Koronakis et al.,2004)

MexXY operon efflux is found to be accountable for aminoglycoside resistance in *P. aeruginosa* strains which were characterized as aminoglycoside impermeability-type resistance (AGIR), Deletion of *MexXY* from wild type*P. aeruginosa* increased their susceptibility to antibiotics of aminoglycoside (Islam et al.,2009) *MexY* is a drug proton anti porter associated with the membrane fusion or linker protein *MexX* and homologous to *AcrD* in E. coli (Maseda et al.,2010)and (Muller et al.,2007).

The advantage of having the inducible pump expressed constitutively in *P. aeruginosa*is the elimination of lag time for induction of the pump in cell populations intermittently exposed to aminoglycosides (Medical Laboratory Manual for Tropical Countries 1991) Considering that the efflux pumps play a significant role in an-tibiotic resistance for *P. aeruginosa*, and the different expression levels in efflux pump genes for various isolates conferring different antibiotic susceptibility pattern, the aim of this study was to determine the expression of the genes encoding these efflux pumps using quantitative Real-Time PCR (RT-qPCR).

MATERIALS AND METHODS

Sampling:

Between September and December 2017, 95 samples were taken from two general hospitals of Baghdad. The swab samples were taken from patients including, burns, wounds, the clinical swabs were collected from tools of burns unit, CSF, inflamed ear, sputum and urine samples from people with inflamed of the urinary tract.

Phenotyping identification of P. aeruginosa:

Bacteriological cultures were performed according to Chees brough (Biochemical Tests for Identification of Medical Bacteria 2000) Identifications of *Pseudomonas species* were done according to colony morphology, characteristic pigment production of *P. aeruginosa,* gram staining, the biochemical test done according to MacFaddin (Bauer et al.,1996), and API 20 E (Collee et al., 1996).

Antibiotics susceptibility test:

Antibiotic susceptibility testing of *P. aeruginosa* was performed according to the Kirby-Bauer disk diffusion method (Clinical and Laboratory Standards Institute2011).The used antibiotics discs were: gentamicin (10 µg).

Plates were incubated at 37 ºC for 18 hrs. The diameters of inhibitory zones were measured and the results were reported based on the recommendation of (CLSI) 2011 (Dumas et al., 2006)**.**

Purified colonies of isolated *P. aeruginosa* were subculture don Brain Heart Infusion (BHI) broth containing glycerol and kept frozen at -70ºC for molecular study.

Genotyping identification of P. aeruginosa:

Bacterial DNA extraction:

Fifty four isolates chosen for DNA extraction according to instructions of Gene aid Company (DNA miniprep kit)**.**

Molecular detection for rpsl gene by PCR assay

*Rps*l gene was used as housekeeping control gene. The method used for amplification and the primer sequence described previously by Dumas et al.,2006 and (Quale et al.,2006) Primer sequences used in this study are listed in table I.

PCR was run under the conditions were listed in table II. (Xavier et al., 2010) and (Livak et al., 2008) the amplified product size were 201 bp.

Molecular detection for MexX gene by PCR assay

PCR was used for amplification of *MexX* gene. Primer sequence used in this study are listed in table I. (Quale et al.,2006)The amplified product size were 326 bp .PCR was run under the conditions were listed in table II (Xavier et al.,2010) the distilled water (as blank template) was used as negative control.

TABLE I: Primers used for PCR assay in this study

Primer	Forward	Reverse	Product size bp	Reference
rpsl	5`GCAAGCGCATGGTCGACAAGA3`	5`CGCTGTGCTCTTGCAGGTTGTGA3`	201	Dumas et al.,[19]
Mex X	5`TGAAGGCGGCCCTGGACATCAGC3`	5`GATCTGCTCGACGCGGGTCAGCG3`	326	

Table 2: The conditions of PCR reaction for primers

PCR gene	Initial denaturation	No. of cycles	Denaturation	Annealing	Extension	No. of cycles	Final extension	No. of cycles
Rpsl	95ºC/5 min	1	94ºC/30 sec	57ºC/30sec	72ºC/1min	30	72ºC/7min	1
mexX	95ºC/5min	1	95ºC /20sec	60ºC/20sec	72ºC/30min	40	72ºC/5min	1

PCR products and 100-bp DNA size marker (Bioneer,Korea) were run simultaneously on 1% agarose gel stained with0.5µg ethidium bromide (Biobasic,Canada) at 70 volt for 90 min in 1X TBE buffer. Finally, the agarose gel was visualized by using UV transllluminator.

Gene expression:

RNA extraction and synthesis of cDNA:

RNA was extracted using RNA extraction kit (Gene aid, Korea) following the protocol provided by the manufacturing. To determine the expression of *MexX* gene for different isolates, Reverse transcriptase Real Time PCR (RT-qPCR was performed for cDNA synthesis using hexamer primer (Wizbio,Korea)(Table III).

TABLE 3: Cdna synthesis program of *rpsl* and *mexX* gene expression

	Step 1	Step 2	Step 3	Step 4
Temperature	25 ºC	42°C	85 ºC	4 ºC
Time	10 min	30 min	5 min	∞

The *rpsl* gene was used as housekeeping gene.the volume of reaction was 20*µl*.Thermal cycler steps of conditions cDNA Reverse Transcriptase showed in Table IV.

TABLE 4: Conditions of primers thermal cycler steps for cDNA reverse transcriptase

Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	5 minutes	1
Denature **Anneal**	95 °C 60 °C	30 seconds 60 seconds	40
Melting curve Analysis	65-95 °C	2-5 seconds/step	1

Real-time PCR assay by usingfluorescent dye SYBER green

Gene exprision of efflex pump gene mexXusing the RT-qPCR assay working with Syber green provided from Wis bio (korea).

Quantititive Real-time PCR. According to protocols,Syber green, primers and synthesized cDNA were mixed in proper volume (table V) and examined by using the QIAGEN Real-time PCR System ((Rotor- Gene Q, Germany) with qPCR soft software. The gene expression levels and fold change were quantified by measuring the threshold cycle (Ct).

The gene expression was compared with *rpsl* gene expression as the housekeeping gene. The ΔΔCt was used for determining gene expression. The ΔΔCt was earned by subtracting ΔCt of mexX from ΔCt of rpsl gene .To the gene expression levels between different samples; the $2^{-\Delta\Delta Ct}$ method was used (Zinsser,1992.)

Table 5: Component of quantitative real-time pcr used in *mexX* and *rpsl* genes expression experiment

Component	Volume per 20µl Reaction
qPCR Master Mix, (Syber green)	10 µl
Forward Primer	1 µl
Reverse Primer	1 µl
cDNA Template	4 µl
Nuclease-Free Water	4µl

RESULTS AND DISCUSSION

Antimicrobial susceptibility pattern of isolates:

The results of antimicrobial susceptibility testing indicated of in (Table 6). The results of gentamycin (GN) resistance showed that 40 isolates (74.07%) were resistant, 8 isolates (14.81%) were intermediate, and 6 isolates (11.11%) were sensitive to gentamicin Moreover, all the tested isolates have high significant differences (P<0.01) for the three levels of susceptibility patterns: resistant, intermediate and sensitive isolates, as shown table 6.

Table 6: Number and percentage of antimicrobial susceptibility rates for pseudomonas *aeruginosa* isolates against gentamicin

Antibiotic	Resistant	Intermediate	Sensitive
GN	40 (74.07%)	8(14.81%)	6(11.11%)
Chi-Square (χ^2)	12.622 **		

(GN) Gentamicin. ** (P<0.01).

Table. VII Number and percentage of antimicrobial susceptibility rates for *pseudomonasaeruginosa* against gentamicin according to sources of isolates

Source of isolates / Antimicr-obialsusceptib-ility	Burns	CSF	hospital equipments	Wounds	Sputum	Ear swaps	Urine	Total
Resistant	14(100%)	1(100%)	1(100%)	5(71.42%)	10(66.66%)	7(58.33%)	2(50%)	40
Intermediate	0 (0%)	0(0%)	0(0%)	1(14.28%)	2(13.33%)	4(33.33%)	1(25%)	8
Sensitive	0 (0%)	0(0%)	0(0%)	1(14.28%)	3(20%)	1(8.33%)	1(25%)	6
Total	14	1	1	7	15	12	4	54
Chi-Square (χ^2)	15.00 **	15.00 **	15.00 **	13.05 **	11.28 **	10.75 **	10.18 **	---

** (P<0.01).

All burns isolates were with full resistance for gentamicin (n=14/14,100%) followed by wounds isolates (5/7, 71.42%), sputum swabs (10/15, 66.66%), inflamed ear (7/12, 58%), and the lowest resistance for urinary tract infections was (2/4, 50%). Statistically, all isolates of *Pseudomonas aeruginosa* for all tested isolation sources were highly significant (P<0.01), as shown in table (VII).

Pseudomonas aeruginosa is a common human pathogen considered as the third agent of nosocomial infections after *Staphylococcus aureus* and *Escherichia coli* (Poole, 2000).One of the main problems regarding treatment and prevention of *P. aeruginosa* infections is the development of antibiotic resistances (Lomovskaya et al.,2001).The efflux pump has been recognized as one of the significant complexes that result in resistance to most antibiotic classes.(Rakesh et al.,2012).

Many investigations have been performed on antibiotic resistance in *P. aeruginosa* with different results depending on time and number and source of sample. The results of this study showed the antibiotic resistance to gentamicin was (74.07% ,n=40) which are partly similar to reported antibiotic resistance values for gentamicin 63%, by Rakesh, et al., in India. (Fazeli et al., 2008) and (AL-Mayyahi 2018)

Also in a study acheived by AL-Mayyahi ; in Was it, the results were 48% for Gentamicin (Anil, and Shahid,2013).and the results of a study by Chander Anil, et al., 2013 in Nepal, resistance to antibiotics was reported for gentamicin was 37.2% ; in which antibiotic resistance rates to gentamicin were lower than this study(Shahcheraghi et al.,2003).In another study performed by Shahcheraghi, et al., 2003 the rates of resistance to gentamicin, was 93.7%, it seems that the reason for higher resistance rates in comparison to the present study (100%) may be associated with the type of clinical samples that was only burn samples which according to previous studies have usually higher antibiotic resistance rates than other clinical samples [30]. In the research done by (Dubois et al.,2008) (Turnidge 2003) they illustrated that the rate of resistance for gentamicin reached to 55.8%and these results are much lower than the percentage of this study.

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 (Poole, 2005).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 (Giedraitienė et al.,2011) and (Al-Jubori et al.,2015)

PCR study for genes:

Confirmatory test which provide a rapid diagnostic identification of bacteria. (Al_Jubori et al., 2015) and (Auda et al.,2012) used *rpsl* gene for detection the same bacteria and reported positive result for all isolates. Figure (1) shows positive agarose gel electrophoresis results for *rpsl* gene products (amplified).

PCR study for efflux genes revealed the presence of genes in 100% of P. *aeruginosa* isolates (Figure 1) The presence of efflux genes was associated significantly with high resistance to gentamicin, *Pseudomonas aeruginosa* represents a common nosocomial pathogen causing various types of infections. This pathogen is known for its association with resistance to a wide spectrum of antibiotics (Poole, 2000).Figure (1) shows positive *P. aeruginosa* isolates for *rpsl* and *MexX*by PCR.

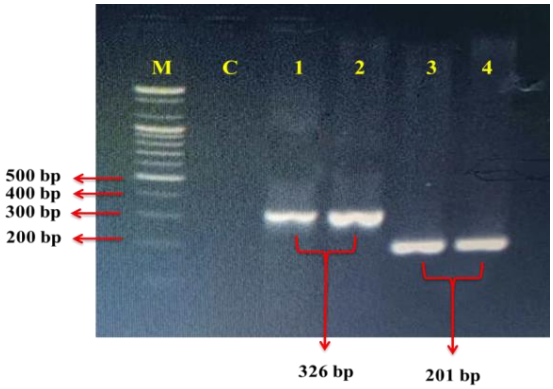

Figure 1. Gel electrophoresis of amplified PCR product of *rpsl* gene(201bp) and MexX gene in monoplex PCR at 70v for 90 min in 1 % agarose , TBE (1x) , stained with ethidium bromide . Lane(M): DNA ladder (100bp),lane (C): negative control(without DNA) , the lanes(1,2):MexX gene (326bp) and the lanes(3,4):*rpsl* gene(201bp).

Molecular investigation using PCR for surveying efflux pump *MexX* genes was showed

that all resistant isolates (n=40) had the *MexX* gene figure (1). Similar results were obtained in a study by Al-Grawi et al., (Radonic et al.,2004)

Twenty eight isolates were selected to detect the presence of AG resistance mechanisms (efflux system genes).

MexX gene was detected in 25 (89.5%) isolates in study performed by Al-Jubori et al., 2015 and (Auda et al.,2012) and this result is approximated in line of the current stud.

Real time PCR quantification of rpsl gene expression:

Rpsl:

The genes of references or housekeeping appear as the normalizing real-time PCR. The reference genes or housekeeping genes are expressed in a wide variety of bacterial cells and show no or only minimal changes in expression levels between the samples and experimental conditions. These genes are used to normalize the mRNA levels of genes of interest before the comparison between different samples by the real time PCR. The right choice is crucial to properly analyze the results of qRT-PCR (eters et al.,2004) . These reference genes are responsible for measuring and reducing the errors from variations among the samples, extraction and RNA quality and efficiency in cDNA synthesis, internal controls and the different experimental samples (Hassan et al.,2012) .To further improve this and although there was a significant difference in the mean Ct value between sources of isolates in the present study, the variation of total change in expression of *rpsl* was studied in different clinical isolates utilizing the 2-Ct value and the ratio of 2-Ct of the different study groups to that of control group, as shown in (Table VIII). Small variations were showed in gene fold expression between the study groups renders *rpsl* gene a useful control gene.

Realtime PCR quantification of MexXexpression:

To assess the possibility of using RT-qPCR to detect antimicrobial resistance mechanisms in *Pseudomonas aeruginosa,* we measured gene expression of the MexXefflux pumps.

All resistance isolates had efflux pump *MexX*gene,but different expressions were observed in different isolates (figure 2) .In addition, there was high level of *MexX* gene expression in bacteria which isolated from burns and the lowest for hospital tools. The results appeared there was a significant difference according LSD-values at $p<0.01$ in different isolation sources, as expline table (IX).

In the past decade, several PCR formats and other molecular methods for the detection of *P. aeruginosa* have been developed. Some studies found a higher sensitivity of PCR in comparison with culture and /or biochemical tests for the detection of *P.aeruginosa* from samples of patients by using real time PCR (Heyrman, 2006) and (Ozer et al.,2012)

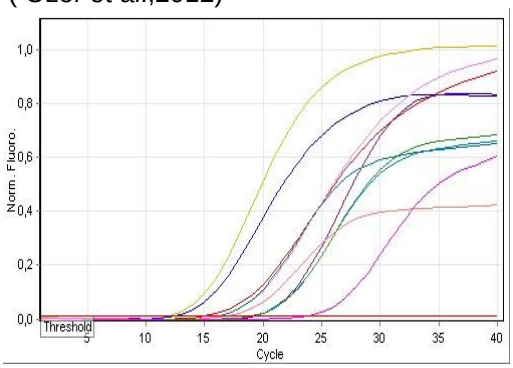

Figure 2. RT-qPCR results showed *MexX* gene expression in *P.aeruginosa*

Ozer et al., investigated survey of efflux pump gene expression of *P aeruginosa* in 50 clinical strains isolated from ICU patients. Their investigation included multiplex PCR assay for determining the four known genes expression of efflux pump as well as *MexX* gene.It showed that efflux pumps were in relation with gentamicin resistance , which illustrated that the prevalence rate of *MexX* was 4% and this result disagreed with the current study(Aghazadeh et al.,2014)

Therefore, it is elusive to precisely describe the fact that efflux pumps expression leads to development of antibiotic resistance. In fact, it seems there is an association between multidrug resistance and efflux pump complexes; in other words, the efflux pumps may confer and/or develop resistance to various antibiotic classes.

A study by Aghazadeh, et al., among(21) CF isolates, *MexA* overexpression was the most prevalent mechanism (47.6%) followed by *MexX(*42.8%), *Amp*C (9.5%), and *OprD* down regulation (4.7%) among 27 burn isolates, the prevalence of *MexX, MexA, AmpC* over expression, and *OprD* down regulation was 62.9%, 74%, and 11.1%, 14.8%, respectively. (Deepak et al.,2012)

In our study, some isolates had extensive resistance to gentamicin but efflux pump gene expression of these isolates were very low ,while

other isolates with high expression; therefore, the related occurred resistance can be attributed to mechanisms other than the efflux pump. The other mechanism associated with antibiotics resistance in *P. aeruginosa* is increased expression of efflux systems *MexAB-OprM and MexXY-OprA.,* repression or inactivation of porin*OprD* and this limit the antibiotics entrance to the bacterium cells (Shahcheraghi et al.,2003)

Table. 9: ct values and fold of gene expression of housekeeping gene *rpsl* and *mexx* for pseudomonas *aeruginosa* clinical isolates

		Sample Treated with antibiotic			Calibrator Untreated with antibiotic				
Is. No.	Source of isolates	Ct of target *MexX*	Ct of reference *Rpsl*	ΔCt	Ct of target *MexX*	Ct of reference *Rpsl*	ΔCt	ΔΔCt	Relative quantification (Folds)= $2^{-\Delta\Delta Ct}$
1	Burns	18.73	16.36	2.37	21.07	16.32	4.75	-2.38	5.20 ± 0.49 a
2	Sputum	19.01	16.33	-2.68	23.77	19.34	4.43	-1.75	3.63 ± 0.18 b
3	Ear	18.23	16.42	1.81	18.78	15.67	3.02	-1.21	2.31 ± 0.11 bc
4	Urine	17.33	16.06	1.27	19.01	15.98	3.03	-1.31	2.47 ± 0.09 bc
5	Wounds	18.19	16.42	1.77	19.86	16.48	3.41	-1.64	3.12 ± 0.27 bc
6	CSF	19.53	17.00	2.53	21.26	17.21	4.05	-1.52	2.86 ± 0.15 bc
7	Hospital tools	17.69	16.98	0.71	18.65	17.25	1.40	-0.69	1.61 ± 0.04 c
LSD value		--	--	--	--	--	--	--	1.726 **

Also the ability of *P. aeruginosa* to formation of bio films.

CONCLUSION

In conclusion, considering the present study and also other performed similar studies, it can be concluded that *P aeruginosa* use various mechanisms to avoid antibiotics and other toxic molecules. *MexX* the most significant pump among the known efflux pumps mechanisms. However, the expression level of this gene is not always the same. Although the function of efflux pump plays an important role in reducing the susceptibility to various antibiotics, the roles of other involved agents and mechanisms in resistance development should not be ignored.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

ABG designed experiments and reviewed the manuscript, bacterial collection, and data analysisand. All authors read and approved the final version.

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