Molecular Study of Efflux *MexX* Gene in *Pseudomonas aeruginosa* Isolated from Iraqi Patients

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Abstract: Clinical isolates (95) collected from patients suffering different infections from teaching hospital in Baghdad\ Iraq. These isolates cultured on specific media to grow *Pseudomonas aeruginosa* only . The growed isolates (54) were diagnosed using classical methods and the API 20E followed by molecular detection using a housekeeping gene (*rpsL*). Fifty four isolates of *Pseudomonas aeruginosa* tested against gentamicin. These isolates were selected to determine resistance mediated by *MexX* efflux system using polymerase chain reaction (PCR) . Out of 54 *P.aeruginosa* isolates, 48(88.8%) gave positive results for efflux system *MexX* (resistance and intermediate isolates), while sensitive isolates 6 (11.2 %) have no *MexX* gene . This may indicate the prevalence these type of resistance in the current isolated bacteria.

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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).Gentamicin (GN) are bactericidal antibiotics that are widely used in treatment for severe infections diseases caused by Gram-negative and Gram-positive bacteria (3). Gentamicin molecules bind to the bacterial 30S ribosomal subunit rendering them unavailable for translation then cell death. Several gentamicin resistance mechanisms have been recognized in different bacterial species including: active efflux system , inactivation of the drugs by gentamicin modifying enzymes , alteration ribosomal target site and 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 *mexX gene* induction, as antimicrobials exported by *MexXY-OprM* that do not target the ribosome was unable to induce *MexX gene* expression and the induction only occurs in the presence of ribosome-inhibiting antibiotics (6). *MexXY-OprM* has a role in the resistance to gentamicin 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 GN resistance mechanisms (efflux system genes) using PCR and studying their prevalence with resistance .

Material and Methods:

Collection Clinical Camples:

Clinical isolates (95) of were obtained from patients suffering various infections from different teaching hospital in Baghdad\Iraq during the period between Sep 2017 till Dec 2017 . These samples were distributed as (9) isolates from urinary tract infections, from burns (15) isolates , from wounds infections (12) , (16) isolates from ear , (40) isolates Sputum from patients suffering from respiratory tract infection,(1) isolate from CSF and (2) isolate from Hospitals' tools

Fifty four isolates were diagnosed as *P.auroginosa* using Api 20E KIT.

Antibiotic Susceptibility Test:

Minimum inhibition concentration (MIC) for all the isolates were determined by the two fold serial dilution methods according to the CLSI (9). The used aminoglycoside antibiotic was gentamicin .

Molecular Detection for Isolates:

Molecular detection was used as follow:

DNA Extraction

Bacterial cell were prepared for fifty four isolates as follow: five ml overnight cultures were prepared in broth media from fresh single colony. Cells were harvested in a centrifuge for 5 min at 6000 rpm. Then the cell pellets were re-suspended in 1ml of sterile water. The resuspended cells were re-centrifugated at 12,500Xg for 15min. The pelleted cells were then used for DNA extraction followed manufacturer instructions for geneaid DNA miniprep kit.

The final purified DNA quantitatively subjected to determine both concentration by (ng per µl) and purity by the ratio of dividing 260/280nm readings were measured via Nano Drop spectrophotometer device.Then, Qualitatively detection of total bacterial DNA was performed by 1% agarose gel using gel electrophoresis at 70V for 30 min . Desktop Gel Imager according to Sambrook and Russel (10).

Detection *rpsL* and *Mex X* genes using PCR:

RpsL gene (a house keeping gene) was used for bacterial diagnosis. Specific primers for *rpsL* and *MexX* gene(11,12) were listed in (table 1). The amplified product size were 201 bp and 326 bp respectively . PCR was run under optimized conditions (table 2) (12).

PCR mixture composed from ready master mix PCR , 5 µl template DNA, 2 µl from each forward and reverse primers for each gene (final concentration 10pmol), then the volume was complete to 20 µl of nuclease free water for each gene, PCR products were electrophoresed in 1% agarose gel and visualized under UV light(10).

Table (1): The sequence of primers used in this study

Primer	Forward	Reverse	Product size bp	Reference
RpsL	5`GCAAGCGCATGGTCGACAAGA3`	5`CGCTGTGCTCTTGCAGGTTGTGA3`	201	Xavier *et al.,* (11)
Mex X	5`TGAAGGCGGCCCTGGACATCAGC3	5`GATCTGCTCGACGCGGGTCAGCG3`	326	Dumas *et al.,* (12)

Table (2): Condition of PCR reaction for Primers .

PCR gene	Initial denaturation	cycle	Denaturation	Annealing	Extension	cycle	Final extension	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/30sec	40	72ºC/5min	1

Results and Discussion:

A. Sampling:

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(13). In this study, fifty four   isolates of *P.aeruginosa* were collected from different clinical samples. Table (3) shows the source and percentage of these isolates.

Table (3): Number and percentage of *Pseudomonas aeruginosa* isolates according to specimens source.

Source of specimens	No. of samples (%)	No. of *P. aeruginosa* (%)
Wounds	12 (12.63%)	7(12.97%)
Urine	9 (9.47%)	4 (7.41%)
Burns	15 (15.79%)	14 (25.92%)
Sputum	40 (42.10%)	15 (27.78%)
Ear	16 (16.84%)	12 (22.22%)
Hospitals' tools	2 (2.11%)	1 (1.85%)
CSF	1 (1.052%)	1 (1.85%)
Total	95 (100%)	54 (100%)

Out of 95 clinical specimens of wounds, urine, burns,ear,CSF, sputum and Hospitals' tools, 54 isolates were positive for *P.aeruginosa* . These positive isolates were obtained in high percentage; 27.78% (n=15) from sputum specimens; while the percentage of burn specimens was 25.92% (n=14), ear swap specimens constituted 22.22% (n=12), wound specimens 12.97% (n=7), urine specimens 7.41% (n=4) and the low percentage was obtained from Hospitals' tools andCSF specimen which achieved 1.85% (n=1) for each.

As shown above, most *P. aeruginosa* isolates collected from burns patient, so this bacterium was considered the major agents of nosocomial Infections in burn unit. These results d i s agree with Iraqi research by Al-ammary and Jabbar (14,15) fromBurn's patients = 66.6% , 60%) respectively. AL-Shamaa (16) which showed with regard to isolates of *P. aeruginosa* isolates the largest shear

were from burns 25/31 then wounds 6/31 of whole clinical samples (74 total isolates/111total samples) had been collected from Educational Al-Yarmouk and AL Kadhimiya hospitals in Baghdad. The study compatible with another Iraqi investigation (17), when found that total of 50 different human specimens isolated from Al-Diwanyia hospital, the number and percentage of *P. aeruginosa* isolates were highest from burns14/20 (70%), followed by otitis11/16 (68%) and wounds 6/14(42%).

This variation of the presence. of *P.aeruginosa* among the infected isolates may attributed to major factors including the differences in the type of samples, method and season of sampling, number of collected samples, sex and age of patients , the geographical area that the samples were obtained from and other conditions that differ among studies of this type (18).

B. Molecular detection:

DNA extraction:

The results showed that, using this protocol was very efficient for DNA extraction from *P. aeruginosa,* since good yields of genomic DNA were obtained (Figure 1).
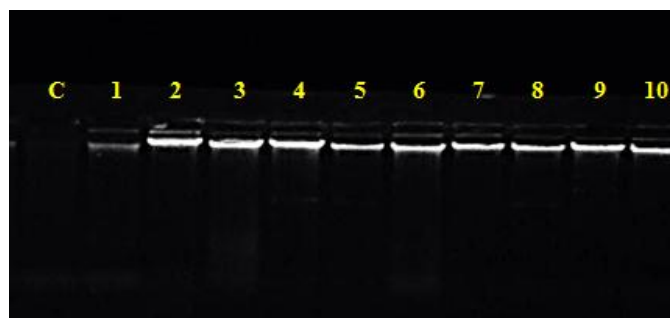

Figure (1): Agarose gel electrophoresis of extracted DNA to check purity and integrity. Lane 1-10: DNA of different *Psedomonas aeruginosa* isolates, Lane C: Negative control. (70 V/ 30 min).

DNA concentration and purity were measured by Nanodrop spectrophotometer, all the isolates had DNA concentration between (50-100 ng/μl) and purity of the DNA were (1.6 - 2).

Antibiotic Susceptibility Test:

Gentamicin are widely used in clinical settings, especially for treatment of life-threatening infections caused by Gram-negative bacteria (19,20). Antibiotic Susceptibility showed that these isolates have very high rate of resistance to antibiotic .

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 most of the isolates 88.8% showed their resistant to gentamicin.

In the research done by Dubois *et al,*.(22) they illustrated that the rate of resistance for gentamicin reached to 55.8%and these result are much lower than the percentage of this study. On the other hand 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 methylation mechanism (24,25).

3. Detection *rpsL* and *MexX* gene using PCR:

Because standard phenotypic methods are time consuming and most have inherent limitations(26). Molecular 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* ,The resultes showed 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* were seen. This technique used internal standards, mainly housekeeping genes, so called because their synthesis occurs in all cell types since they are necessary for the cell survival. Molecular detection of *rpsL* gene by PCR is one of the most commonly used techniques for bacterial identification. Although the *rpsL* gene had the ability to identify most of the isolates at the species level but it is not polymorphic enough to give a clear and specific differentiation among all *Pseudomonas* species, (27). The findings of the current study proved that PCR amplification using *rpsL* gene was a perfect method for the detection of the isolates of *Pseudomonas* spp., Figure (4-6). Al-Jabiri and Al-Jubori(28) used *rpsL* gene for detection the same bacteria and reported positive result for all isolates .as showen in figure (2).
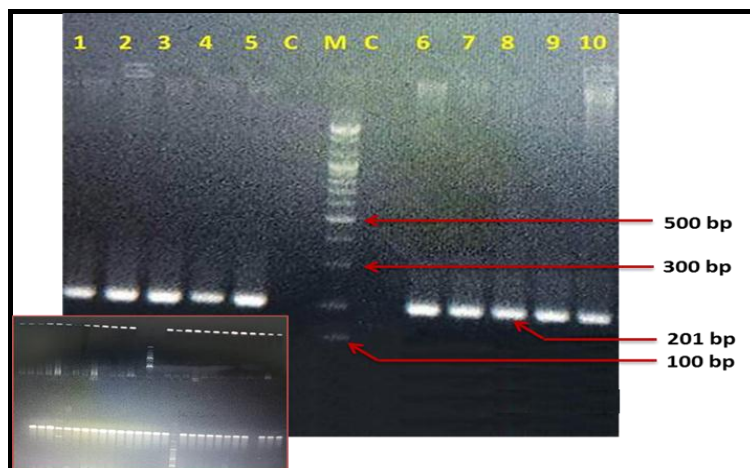

Figure (2): Agarose gel electrophoresis (2% agarose, 70 volt for 90min) of *rpsL* gene PCR product (201bp amplicon). Lane M ,100bp DNA ladder, (1-10) lanes positive results .Lane C,negative control.

Fifty four isolates were selected to detect the presence of GN resistance mechanism . *MexX* gene was detected in 48 (88.8%) isolates and 6 (11.2%)

isolates were negative for presence of *MexX* gene ,molecular investigation using PCR for surveying efflux pump *MexX* genes was showed that all

resistant isolates had the *MexX* gene. Similar results were obtained in a study by Al-Grawi *et al.*, (29).

In study performed by Al-Jubori *et al.*(28) 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 and this result is approximated in line of the current study. 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(30).

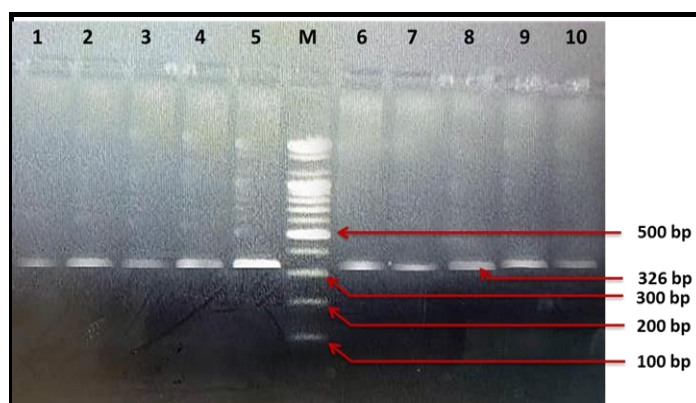Figure (3): Shows agarose gel electrophoresis for efflux system gene (*MexX*).

Figure (3): Agarose gel electrophoresis (2% agarose, 70 for 90min) for *MexX* PCR products (326bp). lane M 100bp DNA ladder. Lanes (1-10): *MexX* product.

The association of efflux genes and resistance to various antibiotics like gentamicin, Imipenem and meropenem has been described in previous studies (31,32).

The efflux pumps play a significant role in multiple antibiotics resistance among *P. aeruginosa* species. These structures act by increasing the MIC concentrations of bacterial species, reducing intracellular antibiotics concentrations thus leads to the appearance of resistant strains.

The therapeutic application of efflux pumps inhibitors is a hope for development of new antibacterial therapy among different bacterial species due to the significant structural homology of efflux pumps. Researches have been concentrated on *P. aeruginosa Mex* efflux pumps and their inhibitors. In previous studies, the inhibitor had lowered the MIC values of fluoroquinolones for both sensitive and resistant strains (33, 34).

In conclusion out of 54 isolates, 48 (88.8%) were harbored at least one GN resistant 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.

The present study highlights the importance of genes controlling efflux pumps as an important cause associated with *P. aeruginosa* resistance to antibiotics. Longitudinal large scale

studies are required for further analysis of these genes and its expression effects on antibiotics resistance of *P. aeruginosa*.

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