EurAsian Journal of BioSciences
Eurasia J Biosci 14, 2811-2816 (2020)

Molecular detection of virulent *exoU* mutation of *Pseudomonas aeruginosa* isolated from wound and burn samples

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Abstract

Pseudomonas aeruginosa is one of the Gram-negative bacteria widespread everywhere in the Pseudomonadaceae family and is able to survive in a wide range of environments. The aim of this study was to determine of virulent *exoU* genotype and phylogenetic tree of *P. aeruginosa* isolates using real time PCRand Sanager sequencing. 206 clinical samples were collected from wounds and burns from both sexes. Samples were cultured on MacConkey agar, Blood agar and Cetrimide agar in order to obtain the bacterial isolates depending on their phenotypic characteristics; biochemical tests as diagnosed with Api 20E; Microgen GnA + B-ID and Molecular detection of *exoU mutation of Pseudomonas aeruginosa* isolates by using RT-PCR and Sanger method. The results showed that 12(24%) of (50) isolates were positive for *ExoU* gene.The *ExoU* gene were used to analyze the DNA sequence of local isolates and compare them with some standard global isolates according to the *ExoU* gene available in the GenBank database of the NCBI database. The results showed that the isolates were (98-100%) matched between local isolates and the international standard isolates. The results of the RT-PCR of the *ExoU* gene were used to detect mutations related to *Pseudomonas aeruginosa*, and the transitions showed the frequency of 5 mutations of the *ExoU* gene in all isolates. In addition, there were three types of mutations such as the predicted frame mutation effect was accompanied by only a transition 3, while 4 mutations were shown silent in all isolates. In addition, 2 mutations were shown to be mismatched. Neighbor joining phylogenetic tree for *ExoU* sequences which indicate one cluster divided into two subgroups. Group 1 showed sister group with *P. aeruginosa* strain BA7823 (India) reference strain from GenBank database which was used for determining the mutations and polymorphisms in the local isolates of this study.

Keywords: *Pseudomonas aeruginosa*, *ExoU* gene, toxin, RT-PCR, Sanger methods

AL-Rubaye MR, Yildiztugay E, Uysa A, Mohammed TK, Abdullah HN (2020) Molecular detection of virulent *exoU* mutation of *Pseudomonas aeruginosa* isolated from wound and burn samples. Eurasia J Biosci 14: 2811-2816.

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INTRODUCTION

P. aeruginosa, causes the most common bacterial infection associated with hospital infections in burns and pneumonia that related with ventilation. It is responsible for otitis media, inflammation of the follicle (inflammation of the pelvis), inflammation of the cornea, soft tissue infections (burns wounds and postoperative), diabetic foot infections, urinary tract infections, bacteremia and pneumonia inflammation in cystic fibrosis patients (Liew et al. 2019; Mukadasi, 2018).*P. aeruginosa* has been resistant to a variety of antibiotics. Treatment of *P. aeruginosa* infections has become a major challenge given the ability of these bacteria to resist many of the antibiotics currently available (Lister et al. 2009; Lari et al. 2015). The World Health Organization (WHO) has recently included *P. aeruginosa* resistant to carbapenim as one of the three bacterial species that urgently needed to develop new antibiotics to treat infections (Tacconelli et al. 2017). The *P. aeruginosa* (5.5-7 Mbp) genome is relatively large compared to other serial bacteria, and represents a significant proportion of the important regulatory enzymes for metabolism, transport, and organic organ flow. The enhanced coding capability of *P. aeruginosa* allows for great metabolic versatility and a high ability to adapt to environmental changes (Klockgether et al. 2011). Certain strains of *P. aeruginosa* produce exotoxin *ExoU* toxin, which is

Received: September 2019
Accepted: March 2020
Printed: September 2020

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associated with increased virulence. *ExoU* causes host cell degradation by lipid degradation in the host membrane through phospholipase activity. *ExoU* has been widely described as a cytotoxicity, resulting in the killing of a rapid number of host cells including epithelial cells and neutrophils (Pazos et al. 2017). The Biochemical and genetic of *P. aeruginosa* isolates diagnosis, in addition to therapeutic importance of the certain antibiotics for wounds and burns must study. Therefore, the aims of this study were to: Isolate of *P. aeruginosa* bacteria from patients with inflammation of burns and wounds; Diagnose and identification of *P. aeruginosa* using chemical tests and VITEK 2 system; Detect of *ExoU* gene in *P. aeruginosa* isolates using real time PCR; Detect of *pelF* gene in *P. aeruginosa* isolates using real time PCR and Study the sequencing analysis of *ExoU* genes.

MATERIALS AND METHODS

A total of 206 swab samples were collected from clinical sources including 67 burns and 139 wounds from patients suffering from burns and wounds infections and under the supervision of a medical specialist at City Medicine Hospital/ department of burns in Baghdad city, in the period beginning from January to the end of March 2019.

I-Identification of bacterial isolates:

Pseudomonas aeruginosa isolates were identified and confirmed by conventional microbiological methods:

1- Cultivation of samples

Blood agar, MacConkey agar and Cetrimide agar were used to study the phenotypes of *P. aeruginosa* colonies which including colonial form, shape and color, size, and aroma (Baron et al. 2007). King A medium (Oxoid, England) and King B medium (Oxoid, England) were prepared as described in (MacFaddin, 2000) to detect *P. aeruginosa*.As well as, The Gram stain was used for identification of *P. aeruginosa* in the samples (Baron et al. 2007).

2- Biochemical tests

Biochemical tests were performed for the diagnosis of isolated *P. aeruginosa* bacteria**:**

Catalase test: the test was done by using the catalase reagent 3% H_2O_2 (Collee et al. 1996).

Oxidase test: the test was done by using the oxidase reagent -N,N,N′,N′-Tetramethyl-p-phenylenediamine dihydrochloride (Difco, England) (Collee et al. 1996).

Motility test: the test was done by using motility medium (Collee et al. 1996).

IMVIC test :as described by (Collee et al. 1996).

Grown at 42°C test: the test was done by using nutrient agar plates were cultured by isolated bacteria and incubated at 42°C for 18-24 hr., the positive result is it growth at this temperature (Collee et al. 1996).

Table 1. Forwards and reverse primers used in this study

Primer	Nucleotide sequence (5´ to 3´)	Size
F	GGGAATACTTTCCGGGAAGTT	428 bp
R	CGATCTCGCTGCTAATGTGTT	

Table 2. Components of RT-PCR reaction

Taq qPCR Master mix components	Stock	Final	Volume	
			1	12.05
Master Mix	2X	1X	5	60 µl
Forward primer	10 µM	1µM	0.5	6.05 µl
Reverse primer	10 µM	1µM	0.5	6.075 µl
Nuclease Free Water			3	36.3 µl
DNA	10ng/ µl	10 ng/ µl	1	
Total volume			10	
Aliquot per single rxn 9 µl of master mix per tube and add 1 µl of template				

API 20E identification system (BioMérieux, USA): is standardized system for identification the Enterobacteriaceae and other non- fastidious, Gram-negative rods by depending on using 20 biochemical tests (Collee et al. 1996)**.**

VITEK2 apparatus system (BioMérieux, USA): The isolates were identified as *P. aeruginosa* by conventional methods as well as by the VITEK-2 system, and this done according to the instructions of the producing company. The VITEK2 is a fully automated system that performs bacterial identification.

II-Moleculae detection of *exo*U and *pelf* **genes.**

1- Extraction of genomic DNA

Genomic DNA was isolated from bacterial growth according to the protocol of ABIOpure Extraction. Quantus Fluorometer was used to detect the concentration of extracted DNA in order to detect the goodness of samples for downstream applications. For 1 µl of DNA, 199 µl of diluted Quantifluor Dye was mixed. After 5 min of incubation at room temperature, DNA concentration values were detected. Virulence genes including *exo*U and *pelf* gene were detected using specific primers.

2- Primer Selection and Preparation

Forward and reverse primers that detect *ExoU* (**428** bp) was chosen in regards to methods described by Kaszab et al. (2011), lyophilized primers were provided, dissolved in sterile de-ionized distilled water to provide a final concentration of 100 pmol/µl as recommended by the provider (Integrated DNA technologies, USA), and stored in a deep freezer until use; mentioned primers sequences were shown in **Table 1**.

3-Real time -polymerase chain Reaction (RT-PCR)

It was performed to amplify different fragments of *exoU and pelF* genes under study.

Reaction Setup and Thermal Cycling Protocol

The extracted DNA, primers, and Tag qPCR Master Mix, Nuclease Free Water (Promega, USA), were thawed at 4℃, vortexes to have homogenous contents, a PCR mixture was made in a total volume as described in **Table 2**. RT-PCR reaction tubes were placed in a thermo cycler PCR instrument, DNA was amplified as in the conditions indicated in **Tables 2** and **3**. This test was

Table 3. RT-PCR reaction

RT-PCR program	Tem. (°C)	Time	Cycle
Initial denaturation	95	05:00	1
Denaturation	95	00:30	40
Annealing	55	00:30	
Extension	72	00:30	

Table 4. Number and percentages of *P. aeruginosa* isolates according to the source of isolation by using different culture media

Source of isolate	Number of samples (%)	Number of *P. aeruginosa* isolates
Wounds	67(32.52)	19(38)
Burns	139(67.47)	31(62)
Total	206(100)	50(100)

done according to the instructions of the producing company.

4-Sequencing, Alignment and Phylogenetic Analysis

Sequencing was performed to detect mutations, the RT-PCR of 12 isolates for *ExoU*, was sent to Macrogen Company, USA by using forward primers of *ExoU* genetic analyzer (Applied Biosystems) and using Sanger sequencing method (1977) (Sanger Sequencing Steps and Method, 2020) Homology search was performed using Basic Local Alignment Search Tool (BLAST) program online using blastn algorithms, which are available at the National Center for Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov). Bioedit program (V.7.2) was used for multiple sequence alignment in Clustal W, as well as to calculate the similarity matrix between bacterial isolates groups. To find the relationships between P. aeruginosa isolates groups, phylogenetic analysis was performed using MEGA6 software employing the method of neighborhood-joining (NJ). For estimating the nod branching probability, 1000 bootstraps were used.

RESULTS AND DISCUSSION

The study included the collection of 206 swabs of burn injuries and wounds from both sexes, males and females, ranging in age from 1-70 years, in the period from January to the end of March 2019, from burns hospital in the City of Medicine and Baghdad Teaching Hospital. After the final diagnosis of samples, 50 isolates of *P. aeruginosa* (24.27%) was obtained as shown in **Table 4**.

On MacConkey agar, the bacterial colonies appeared pale yellow because they had not fermented lactose, and this compatible with the results of previous researches (Forbes et al. 2002). On Nutrient agar, the growing of *P. aeruginosa* colonies were identified depending on the pigments and odor production, (grape like odor). On the blood agar medium, the bacterial colonies gave Beta-hemolysis (β). On the Cetrimide agar, the bacterial colonies appeared in greenish yellow, on the King A agar, they produced a blue and green pigment (pyocyanin), while all isolates grow on King B agar did not produce pyocyanin. Microscopic examination showed that they were Gram negative bacilli. In the biochemical tests, all isolates showed positive results for Catalase test, which explained the bacterium's ability to break down hydrogen peroxide into water and oxygen gas. The score IMViC tests was Indole (-), Methyl red (-), Voges-Proskauer (VP) (-), positive (+) result for Citrate consumption, as shown in **Table 2** and **Fig. 1**. Kligler's Iron Agar (KIA) have alkaline interaction (cannot ferment glucose and lactose), H_2S production (-). Growth temperature is 37°C and 42°C, and negative results were obtained for urease test; they were positive for motility test because the bacteria have flagella, these results corresponds to (Tadesse & Alem, 2006; Todar, 2011; Noreen, et al , 2016).

Detection of *ExoU* gene Mutation and Phylogentic trees analysis

Detection of *ExoU* genes was conducted over 12 isolates, using the *exoU* primers by qRT-PCR. In this study, the PCR product of 12 isolates were sequenced, analyzed and compared with the reference strains available in the GenBank database of the NCBI database. By using Basic Local Alignment Search Tool (BLAST), which is available at the NCBI, the sequencing results demonstrated 98-100% compatibility with reference strains as shown in **Table 5** and alignment with references from NCBI. The bit Score is defined as a statistical measure of the moral similarity and the higher value indicated that the high degree of similarity, and if dropped from the class of 50 points, the sense that there is no similarity. Expectation value (E) is defined to give an estimate of the number of times expected to get the same similarity coincidental and lower the E-value. This indicates that the degree of similarity was high between sequences. The value of a very close to zero means that these sequences are identical.

To study the sequencing data for Mutations, DNA sequencing was performed on the PCR products of *ExoU* gene to detect mutations related to the *P. aeruginosa*, the results were illustrated in **Table 5** and **6.**

Transversion and transition showed a frequency of 5 mutations for *ExoU* gene in all isolates. In addition, three types of mutations such as frame shift mutation predicted effect was 3 accompanied with transition only, while 4 silent mutations were shown to be in all the isolates. In addition, 2 mutations were shown to be mismatch.Transitions are interchanges of two-ring purines (A↔G) or of one ring pyrimidine (C↔T), hence involve bases of similar shape; however, transversions are interchanges of purine for pyrimidine bases, hence involve a change of one-ring to two-ring structures and Vis versa, Although there are twice as many possible transversions, because of the molecular mechanisms by which they are generated, transition mutations are generated at higher frequency than transversions due to

Table 5. Sequencing ID in GenBank, score, expect and compatibility of DNA sequences for *ExoU* gene in *P. aeruginosa*

Isolates number	Seq. ID	C.	Expect (E-value)	Identity	+Ve GenBank
Pr01	CP032569.2	24	0.0	100%	4397021 to 4397382
Pr-02	CP032569.2	81	9e-172	98%	4397021 to 4397382
Pr02R	CP032569.2	40	4e-176	98%	4397021 to 4397382
Pr03F	CP032569.2	81	9e-172	98%	4397021 to 4397310
Pr-03R	CP032569.2	40	4e-176	98%	4397021 to 4397382
Pr-04F	CP032569.2	84	4e-145	99%	4397021 to 4397382
Pr-05R	CP032569.2	84	4e-145	99%	4397021 to 4397382
Pr-05F	CP032569.2	10	7e-180	99%	4397021 to 4397382
Pr-05R	CP032569.2	55	0.0	99%	4397021 to 4397382
Pr-06	CP032569.2	24	0.0	100%	4397021 to 4397382
Pr-08	CP032569.2	24	0.0	100%	4397021 to 4397382
Pr-9	Contamination				-
Pr-10	CP032569.2	24	0.0	100%	4397021 to 4397382

Pr: *Pseudomonas aeruginosa* F:Forword R:Reverse

Table 6. Location, nucleotide change, number of codon, amino acid change, and type of mutations in *ExoU* genes of *P. aeruginosa* isolates

Location of gene bank	Nucleotide change	A.a change	Predicted effect	Type of mutation
G/C 4397400	CTC/CTG	L/L	Silent, Framshift	Transversion
G/A4397415	GGG/GGA	G/G	Silent	Transition
C/A 4397416 C/A4397417 G/A 4397418	CCG/AAA	P/K	Mismatch	Transition Transition Transversion
C/G G/A 4397419	CCG/GCA	R/A	Mismatch	Transversion
C/A 4397419	CTC/CTA	L/L	Silent	Transversion
A/G439790	AAG/AAA	K/K	Silent ,Framshift	Transition
C/T 437048 C/G 4307049	TGC/CCG	P/C	Framshift	Transition Transversion

L: Leucine **G**: Glycine **P**: Proline **K**: Lysine **R**: Arginine **C**: Cysteine A.a : Amino acid

Fig. 1. Neighbor-joining phylogenetic tree analysis of *ExoU* for 8 *P. aeruginosa* clinical local isolates arranged in two groups. The analysis performed using MEGA6

tautomeric shift. Transitions are less likely to result in amino acid substitutions due to “wobble”, and are therefore more likely to persist as “silent substitutions” in populations as single nucleotide polymorphisms (SNPs) (Futuyma, 2013) and also transition mutations are less likely to be removed by natural selection because they more often create synonymous substitutions, which encode the same amino acid sequence as the original DNA (Ohno et al., 2014).

Sequence was done using alignment of nucleic acid sequences of reference strain *P.aeruginosa* strain BA7823, to confirm mutations found in our *P. aeruginosa* isolates. Query represents the local sample; Subject represents a database of the NCBI. The nucleotides that are shown in yellow color refer to variation. While the amino acid in pink color indicate amino acid variation.

Fig. 2. Neighbor-joining phylogenetic analysis of *ExoU* for 8 *P. aeruginosa* clinical local isolates compared with international isolates. The analysis performed using MEGA6

The sequence similarity matrix *ExoU* of 8 *P. aeruginosa* clinical local isolates were estimated by Mega 6 program for sequencing nucleotide. The results of the program showed that studied groups of *ExoU* have high sequence similarity ranging between 98-100% (**Table 6**). Phylogenetic analysis was performed in the current study to determine the relationships among studied groups by comparing the *ExoU* sequences obtained in this study with GenBank database sequences in NCBI. **Fig. 2** showed neighbor-joining phylogenetic tree (dendrogram, unrooted) for *ExoU* sequences which indicate one cluster divided into two subgroups. The first subgroup contained all the local isolates (Pr-01,02,03,05,06,08,010) except those isolates that belonged to (pr-04) which were placed in the second subgroup.

Neighbor joining phylogenetic tree (dendrogram, unrooted) for *ExoU* sequences (Figure 2) which indicate one cluster divided into two subgroups. Group 1 showed sister group with *P. aeruginosa* strain BA7823 (India)

reference strain from GenBank database which was used for determining the mutations and polymorphisms in the local isolates of this study.

CONCLUSION

The conclusions from the current study were showed *ExoU* gene in *Pseudomonas aeruginosa* isolates where the results showed that (12) isolates were carriers of the *ExoU* gene. The results of the sequence analysis of the nitrogenous bases of a number of *Pseudomonas aeruginosa* isolates carrying the *ExoU* gene and after matching them with a number of global isolates showed a 98-100% compatibility with the reference strains. DNA sequence results on PCR products of the *ExoU* gene to detect mutations related to aeruginosa showed 5 mutations of the *ExoU* gene in all isolates. In addition, there were three types of mutations such as the predicted frame mutation effect was accompanied by only a transition 3, while 4 mutations were shown silent in all isolates. In addition, 2 mutations were shown to be mismatched.

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