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Study of *pvd* A Gene Sequencing from *Pseudomonas aeruginosa* Isolated from Burn Infections

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Abstract

Pseudomonas aeruginosa possesses two types of iron transport systems and one of them is Pyoverdine, which is fluorescent under the UV light and encoded by the *Pvd*A gene. This pigment is great importance in iron deficiency or its loss affects to cell division and stops the DNA manufacturing process. The other type of pigment is Pyochelin, and that both types are formed during infection with *P. aeruginosa* and tissue invasion in the host's body. The DNA sequence was analyzed for the *pvd*A gene in *P. aeruginosa* that isolated from the burns. The results of this study showed that 15 isolates of the *P. aeruginosa* from 23 isolates have *pvd* A gene at 65.22%, while 8 isolates did not have the *pvdA* gene at a rate of 34.78 %, with molecular weight of 1281 base pairs. Also, the analysis of *pvd A* gene sequencing showed that there are 21 genetic mutations. Include insertion, deletion and replaced.

Keywords: *Pseudomonas aeruginosa*, *pvd* A gene, Mutations.

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Study of *pvd* A Gene Sequencing from *Pseudomonas aeruginosa* Isolated from Burn Infections

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دراسة التحليل التتابعي لجين A *pvd* في بكتريا *Pseudomonas aeruginosa* المعزول من التهابات الحروق

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الخلاصة

تمتلك *Pseudomonas aeruginosa* نوعين من أنظمة نقل الحديد أحدهما هو Pyoverdine، وهو متوهج تحت ضوء الأشعة فوق البنفسجية، ويشفر من قبل الجين A. *Pvd* هذا الصبغة لها أهمية كبيرة في نقص الحديد و يؤثر فقدانه على انقسام الخلايا ويوقف عملية تصنيع الحمض النووي .النوع الآخر من الصبغات التي تمتلكها البكتريا هي Pyochelin، وكلا النوعين تنتج أثناء الإصابة بـ *P. aeruginosa* وغزو الأنسجة في جسم المضيف. تم تحليل التسلسل التتابعي للحمض النووي لجين A *pvd* في *P. aeruginosa* المعزول من الحروق اظهرت نتائج هذه الدراسة أن 15 عزلة من اصل 23 عزلة من بكتريا *P. aeruginosa* تحتوي على جين A *pvd* بنسبة 65.22٪، بينما اظهرت 8 عزلات عدم امتلاكها لجين A *pvd*بمعدل 34.78٪ ، وكانت حزم الجين بوزن جزيئي 1281 زوج قاعدي.

اظهرت نتائج التحليل التتابعي لجين A *pvd* أن هناك 21 طفرة جينية تضمنت الطفرات من نوع اضافة وحذف واستبدال.

الكلمات المفتاحية: *Pseudomonas aeruginosa* ، جين A *pvd*، الطفرات.

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative bacilli, cell diameter between 0.5 -to 0.8 µm and length is 1.5 – to 3 µm. Bacteria cell appears as singles or in the form of pairs or short chains [1], non-spore forming bacteria. It is moved by polar flagella, which is surrounded by a cell wall, the optimum temperature for its growth is 37 °C, but it has the ability to grow at a temperature of 42°C [2]. *P. aeruginosa* is able to secrete many pigments, including the green-blue pyocyanin, and the culture medium of the bacteria acquires the color of the pyocyanin like Muller Hinton agar and nutrient agar, bacteria also have a pyoverdin pigment are greenish-yellow in color and these pigments are dissolved in water [3]. These bacteria grow

on MacConkey agar and appear non -Lactose Fermentation. When growing on the blood agar analyzes blood and this is due to the production of the hemolysin enzyme [4], gives a positive result for the examination of Oxidase and Catalase. *P. aeruginosa* caused many disease like Bacteremia, and most often this disease occurs in patients with immunodeficiency, especially in those with acquired immune deficiency syndrome (AIDS) and cases of wounds and severe burns [5]. As the skin is the body's first line of defense against pathogens attacking the body and, accordingly, the loss of skin to this function after exposure to damage from burns, wounds, or any other cause that results in an increased risk of pathogens having a role mainly in the event of death in wound and burn infection [6]. Also, infection can occur in the urinary tract infection, there are millions of the patients with urinary tract infection every year, these infections often occur in hospitals, especially in developing countries [7]. Catheterization is one of the important factors that lead to urinary tract infections through the catheter tubes that are contaminated with pathogens [8], as well as, respiratory tract infection, which is one of the causes of chronic lung infections associated with ventilator [9]. It causes Endocarditis as it affects the heart valves when using intravenous drugs or using artificial heart valves that are contaminated and thus stabilizes itself in the lining of the heart [10].

Pyoverdine (Fluorescein), which is fluorescent under the UV light and encoded by the *Pvd A* gene. There are other genes that encode their production, which is expressed in conditions of iron deficiency in the bacterial cell [11, 12]. In order to infect and grow in mammalian tissue, *P. aeruginosa* dependent on the bacterial acquisition of iron from proteins that bind mammalian iron. Transferrin is one of the most important iron binding proteins in host defense. The most common mechanism by which bacteria compete for iron with transferrin is via siderophores which is a bacterial product that bind iron, and function in high-affinity iron transport feature [13]. *P. aeruginosa* possesses two types of iron transport systems and one of them is Pyoverdine. Therefore, the pigmentation of Pyoverdine is of great importance because iron deficiency or its loss affects the cell division process and stops the DNA manufacturing process [14]. The other type is Pyochelin, and that both types are formed during infection with *P. aeruginosa* and tissue invasion in the host's body [15].

Aims of this study was genetic detection of *pvd* A gene in *P. aeruginosa* by using PCR technique and study the sequencing analysis and mutation.

Materials and Method

Bacterial isolation

Burn swab samples were collected from several hospitals in Baghdad city (Central Children's Hospital, AL-Imammain AL-Kadhimain Medical City Hospital, Ibn Al-Baladi, Al-Sadr, and Educational Laboratories and Burns Hospital / Medical City).

Diagnosis of isolates

MacConkey agar, Blood agar (for detection hemolysin), Citrimide agar and Pseudomonas agar were used for morphological diagnosis. In addition, biochemical tests such as Oxidase and Catalase were done. Bacteriological tests were confirmed using API 20E for final diagnosis [16]. After the confirmation of isolates diagnosing, we obtained [23] isolation of *P. aeruginosa*. Isolates were diagnosed using *16sRNA* methods in a previous study.

DNA Isolation

DNA was extracted from bacteria isolates using a DNA extraction kit (Geneaid Biotech kit system, UK) according to the manufacturer's instructions.

DNA purity

The purity of DNA was measured using Nano-drop, by applying 1µl of the extracted DNA in the sampling port of the device. DNA samples with purity range of 1.7 and 2 were included.

Detection of the *pvd A* gene

Primers used in this study *Pvd* A (F: 5'GACTCAGGCAACTGCAAC3') (R: 5'TTCAGGTGCTGGTACAGG3') (Size 1281 bp) Cotar *et al*. (2013) [17]. The working solution was prepared at a concentration of 10 picomol / microliter (by taking 10 microliter of the stock solution and adding 90 microliter of ionic distilled water) and stored with the stock solution for the precursors at - 20°C, taking into account mixing the working solution after taking it out ice using the mixer Vortex Griffin (England) to homogenize it before use [17].

The PCR reaction mixture was prepared for the pvd A gene which consist of ready to use GO Taq Green Master Mix (Bioneer, Korea), 5 microliter of extracted DNA template, 1.5 microliter of F-Primer, 1.5 microliter of R-Primer and 12 microliter of sterile ionic distilled water (Bioneer, Korea), then the contents mixed well in PCR eppendorf tubes using mixer and then put it in PCR machine. The pvd A gene amplification reactions were carried out according to [17] as in table 1.

The DNA amplification product were separated using electrophoresis on 2 % agarose gel containing 5 microliters of the Ethidium bromide dye (Bio Basic INC, Canada). DNA ladder (100 bp) was loaded into the first well, then 5 microliters of the DNA amplification product were loaded to the other wells. Gel electrophoresis system were turned on and adjusted at 50 volt for two hours then photographed under ultraviolet light (Optima, Japan) [18].

DNA Sequencing analysis

After initial amplification of *P. aeruginosa Pvd*A gene, (20µl) of each of the DNA PCR product, F primer and R primer were sent to US NICEM Company for sequencing by Genetic analyzer. DNA sequence data were analyzed using NCBI (National Center for Biotechnology Information) database and BioEdit program (V.7.2.5) [19].

Table 1: Steps of PCR Device to investigate the *pvd* A Gene

Step	Program	
1	Only one cycle for 2 minutes at a temperature of 95 °C for the primary DNA denaturation.	
2	30 cycle included:	
	A	95 sec at 30 °C for DNA template denaturation.
	B	59 sec at 30 °C for the primers to bind to DNA template annealing
	C	72 sec at 30 °C for the associated primers to be elongated.
3	Only one cycle for 7 minute at 72 °C for the final elongation of the double DNA strip.	

Result and discussion

The results of the current study showed that 15 out of 23 isolates of the *P. aeruginosa* have *pvdA* (65.22 %). While 8 isolates did not have the gene (34.78 %), with molecular weight of 1281 bp, figure 1. These results were consistent with the result obtained by [20], when they found that the percentage of possession of *P. aeruginosa* for the *pvd* A gene was 68%, while [21] found that the percentage of *pvd* A was 89 % however, their study was conducted on isolates of *P. aeruginosa* that isolated from sputum. Pyoverdine pigment encodes by *Pvd* A gene, the product of the *pvd* A gene is expressed when the iron conditions deficiency in bacterial cell, as this pigment is one of the iron transport systems [14].

The iron is limited under aerobic conditions and pH =7 in tissues and fluids of mammalian hosts due to the presence of transferrin and lactoferrin in serum and mucosal secretions, respectively [22]. Therefore, most microorganism including *P. aeruginosa* have develop a high-affinity uptake methods based on the synthesis of low molecular-weight chelators (siderophores). *P. aeruginosa* produce pyoverdin which has a very high affinity for Fe (III) to stimulate their growth in iron-limited growth environment [23, 22].

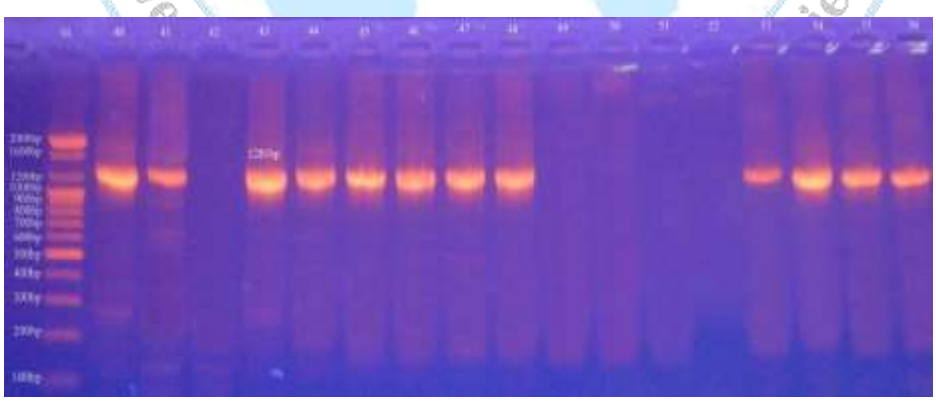

Figure 1: Electrophoresis of the PCR product of *pvdA* gene (1281bp) for *P. aeruginosa* on 2% agarose with Ethidium bromide dye (concentration 0.5 µg / ml). And 50 volts for 2 hr. The M ladder (is ranged from 100- to1500 bp).

In this study the DNA sequence was analyzed for the *pvd A* and showed that there are 21 genetic mutations. Adenine nitrogenous base was replaced by the Guanine nitrogenous base at Position 51 at Subject 2876782 and Guanine was replaced with the Adenine nitrogenous base at Position 118 at Subject 2876713.

Furthermore, Thymine nitrogenous base replaced with Cytosine base at Position 160 at Subject 2876671 and the nitrogenous base Cytosine was replaced with Thymine nitrogenous base at Position 697 at Subject of 2876134. Guanine was replaced with Adenine nitrogenous base at Position 900, 916 and 959 at Subject 2875933, 2876125 and 2875981, respectively. While Guanine nitrogenous base was replaced by Cytosine nitrogenous base at Position 929 at Subject 2876111. Also, Guanine nitrogenous base was replaced by the Thymine nitrogenous base at Position 973 at Subject 2875867. There were two deletion mutation in nitrogenous base Cytosine and Guanine at Position 17 and 37 at Subject 2876818 and 2876796, respectively. Moreover, there were ten mutation of insertion type, three insertion mutation of Guanine at Position 775, 952 and 964 at Subject 2876058, 2875988 and 2875976, respectively, Four insertion mutation of Thymine at Position 892, 901, 932 and 974 at Subject 2875942, 2875934, 2876108 and 2875866, respectively, two insertion mutation of Adenine at Position 921 and 960 at Subject 2876119 and 2875980, respectively and only one insertion mutation of Cytosine at Position 944 at Subject 2875996, table 2 and figure 2 : A and B.

Similarly, Sokol *et al*., (1999) [24] mentioned that the *pvd A* mutants were less virulent than the parent strain in chronic and acute models of respiratory infection. However, the acquisition of iron can play a role in the pathogenesis of *P. aeruginosa*. *Pvd* gene cluster is directly related to the pioverdin synthesis, which is a fluorescent peptide-siderophore involved in the iron acquisition, being essential in the bacterial pathogenicity on the host [25]. A particularly well stud-ied group of siderophores are the pyoverdines (PVDs), pigments and important virulence factors of fluorescentpseudomonads. Almost 70 strain-specific PVDs have beendescribed to date [26]. The concentration of iron in the growth media has a major impact on the extracellular protein, toxin A, alkaline protease and elastase production. Therefore, when grown in human

serum and transferrin, a mutant unable to synthesize pyoverdin had an exceptionally depressed growth rate relative to that of wild-type strains. Pyochelin has also been shown to raise *P. aeruginosa* lethality during infections in mice [27]. Further studies are required to understand the effect of mutation on *pvd* A gene at the expression level at different concentration of iron availability and to find the role of *pvd* A gene mutations on protein translation, pigment production and virulence factor.

Table 2: Genetic mutations in the nitrogenous bases of *pvd* A from *P. aeruginosa*

No.	Nitrogen bases	Changes in Nitrogen bases	Position	Subject	Changes type
1	Cytosine	-	17	2876818	Deletion
2	Guanine	-	37	2876796	Deletion
3	Adenine	Guanine	51	2876782	Transversion
4	Guanine	Adenine	118	2876713	Transversion
5	Thymine	Cytosine	160	2876671	Transversion
6	Cytosine	Thymine	697	2876134	Transversion
7	-	Guanine	775	2876058	Insertion
8	-	Thymine	892	2875942	Insertion
9	Guanine	Adenine	900	2875933	Transversion
10	-	Thymine	901	2875934	Insertion
11	Guanine	Adenine	916	2876125	Transversion
12	-	Adenine	921	2876119	Insertion
13	Guanine	Cytosine	929	2876111	Transition
14	-	Thymine	932	2876108	Insertion
15	-	Cytosine	944	2875996	Insertion
16	-	Guanine	952	2875988	Insertion
17	Guanine	Adenine	959	2875981	Transversion
18	-	Adenine	960	2875980	Insertion
19	-	Guanine	964	2875976	Insertion
20	Guanine	Thymine	973	2875867	Transversion
21	-	Thymine	974	2875866	Insertion

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Figure 2 A: DNA sequences of *pvd A* from *P. aeruginosa*.

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A1_AF

Pseudomonas aeruginosa genome assembly PAO1OR, chromosome : I
Sequence ID: emb|LN871187.1|

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
1657 bits(897)	0.0	947/969(98%)	12/969(1%)	Plus/Minus

Features:
L-ornithine N5-oxygenase

```
Query  13       CGCA-CGGCAGCACCGACAGCAGG-TGTCGCTGAGGCCGTGGCTGGCCTGGCTGAAGCCC  70
                ||||  ||||||||||||||||||  ||||||||||||  ||||||||||||||||||||
Sbjct  2876820  CGCACCGGCAGCACCGACAGCAGGGTGTCGCTGAGGCCATGGCTGGCCTGGCTGAAGCCC  2876761

Query  71       TGCGCGTAGATCGCCACCTTGCAGCGCTCGTCGGTCTGCAGGCGGTAATCGCGGCCGATC  130
                ||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  2876760  TGCGCGTAGATCGCCACCTTGCAGCGCTCGTCGGTCTGCAGGCGGTAGTCGCGGCCGATC  2876701

Query  131      TCGTGGTCGCCGAGGTACTCCGCCAGCGGCTCGAGCAGTTGGCGGTGCAACTGGCGCTCA  190
                |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  2876700  TCGTGGTCGCCGAGGTACTCCGCCAGCGGTTCGAGCAGTTGGCGGTGCAACTGGCGCTCA  2876641

Query  191      TAGCCGGTGGCCAGGATCACTGCGTCGTAGGTCTCTACGCTTAGCTCGCCGCTACCGGCG  250
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2876640  TAGCCGGTGGCCAGGATCACTGCGTCGTAGGTCTCTACGCTTAGCTCGCCGCTACCGGCG  2876581

Query  251      TCGCGCAACGCCAGCTCGATGCCCTGGGCGGTGGCGGTCGCGCGCTCCACGGTGGTCATG  310
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2876580  TCGCGCAACGCCAGCTCGATGCCCTGGGCGGTGGCGGTCGCGCGCTCCACGGTGGTCATG  2876521

Query  311      CAACGGAAGGCGTGGCGCGGGATGCCGGAGACTTTCTGGCGGTAGAAGACGCCGTAGATG  370
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2876520  CAACGGAAGGCGTGGCGCGGGATGCCGGAGACTTTCTGGCGGTAGAAGACGCCGTAGATG  2876461

Query  371      CGCTCGATCAGGTCGGTATCCACCACCGAATAGTTGGTGTTGTGGTATTCGCGCAGCAAA  430
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2876460  CGCTCGATCAGGTCGGTATCCACCACCGAATAGTTGGTGTTGTGGTATTCGCGCAGCAAA  2876401

Query  431      CGCTCGCGTTCGGCATGTTCGCGGCTGTAGATGAGATCGGTGAACTTCGGCGCGAACACT  490
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2876400  CGCTCGCGTTCGGCATGTTCGCGGCTGTAGATGAGATCGGTGAACTTCGGCGCGAACACT  2876341

Query  491      TCGTTGACGAACGGGCTATCGTCCGCCGGCTTGAGCGCCGAGGCACGCAGGATCATGTCG  550
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2876340  TCGTTGACGAACGGGCTATCGTCCGCCGGCTTGAGCGCCGAGGCACGCAGGATCATGTCG  2876281

Query  551      GCCTGCACCGACGGGTAGCTGTCGTTGAGGTCGATGAAGGCCTCCGCCGCGCTCTGCCCG  610
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2876280  GCCTGCACCGACGGGTAGCTGTCGTTGAGGTCGATGAAGGCCTCCGCCGCGCTCTGCCCG  2876221

Query  611      CCGCCGATAATGGCGATCTTCATCGGCTTGCCACTGCTGCAGGGCTGCTTGGCCATGTGT  670
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2876220  CCGCCGATAATGGCGATCTTCATCGGCTTGCCACTGCTGCAGGGCTGCTTGGCCATGTGT  2876161

Query  671      TCCAGGTACTGGCTGTGGTGGAACACTCGGCCGTCGCCCTTGAGCGCACGGAACACCTGC  730
                |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  2876160  TCCAGGTACTGGCTGTGGTGGAACACCCGGCCGTCGCCCTTGAGCGCACGGAACACCTGC  2876101
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Query  731      GGGATACGCGGGGTGCCGCCGGGACTGACCACCAGGGCGCGGGGTGGTGCGCACCAGCTC  790
                ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  2876100  GGGATACGCGGGGTGCCGCCGGGACTGACCACCAGGGCGCGGG-TGGTGCGCACCAGCTC  2876042
Query  791      CTCGCCGTCGGCGTTGCGCGAGATCACCCGCAGCGCCTCGACCTGGCCGGCGCTCAGCAT  850
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2876041  CTCGCCGTCGGCGTTGCGCGAGATCACCCGCAGCGCCTCGACCTGGCCGGCGCTCAGCAT  2875982
Query  851      CGGCTCGATGCGCAGGACCTCTTCGCCGTAGCGGCTCTGCTTCCTGGAAATTGGCTGGCG  910
                |||||||||||||||||||||||||||||||||||||||| ||||||| |||||||||
Sbjct  2875981  CGGCTCGATGCGCAGGACCTCTTCGCCGTAGCGGCTCTGCT-CCTGGAAG-TGGCTGGCG  2875924
Query  911      ACCCAACGCAAGGTAGTCCTTTGAACTCCATCCCGGCAGGGGATAAAAAAGTGGCCCAGG  970
                ||||| |||| ||||||| || |||||||||| ||||||| ||| ||  ||| ||||||
Sbjct  2875923  ACCCAGCGCA-GGTAGTCGTT-GAACTCCATCC-GGCAGGG-ATAGAAG-GTG-CCCAGG  2875870
Query  971      TTTTATGAA  979
                ||  |||||
Sbjct  2875869  TTG-ATGAA  2875862
```

Figure 2 B: DNA sequences of *pvd A* from *P. aeruginosa*

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