Zagazig J. Pharm. Sci. June, 2018 ISSN 1110-5089
Vol. 27, Issue 1, pp. 22- 30 ISSN (on-line) 2356_9786

# Phenotypic and genotypic characterization of *Pseudomonas aeruginosa* siderophores

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## ABSTRACT

*Pseudomonas aeruginosa* is an opportunistic human pathogen that causes many infections such as urinary tract, respiratory tract, burn infections in addition to septicemia. Iron (Fe) is an essential element for the growth of most living microorganisms. Siderophores are iron chelating compounds produced by bacteria. *Ps. aeruginosa* produces two major siderophores; pyoverdine and pyochelin.

*Ps. aeruginosa* isolates were identified morphologically, biochemically and by culture characteristics. Siderophore production was assessed phenotypically by chrome azurol sulfonate (CAS) Shuttle assay and genotypically by PCR.

Siderophore production varied among the clinical isolates. High production was found in 63.33% of isolates. Intermediate production of siderophore was detected in 30%, while 6.66% of isolates showed a low level of siderophores. Genotypic detection of genes responsible for the formation of siderophores and their receptors revealed the presence of *PvdS*, *PtxR and FpvA* genes that are responsible for pyoverdine synthesis and its receptor formation in all isolates. Whereas *pchG* and *FptA* encoding pyochelin and its receptor were detected in 85.7% of isolates.

Siderophores are important iron chelators in *Ps. aeruginosa*. Substantial correlation was received from the genotypic and phenotypic investigations of siderophore production.

**Key words:** *Ps. aeruginosa***,** Siderophores**,** pyoverdine, pyochelin

## INTRODUCTION

*Pseudomonas aeruginosa* is frequently found as a part of the human microflora in healthy individuals. *Ps. aeruginosa* is an opportunistic pathogen for humans that is responsible for a wide range of diseases including infections of the urinary tract, respiratory tract, burn, and septicemia **(Yang *et al.*, 2011)**.

Iron (Fe) is an essential element for the growth of most living microorganisms because it is used as a catalyst in enzymatic processes, oxygen metabolism, electron transfer, and synthesis of both DNA and RNA **(Aguado-Santacruz *et al.*, 2012)**.

*Ps. aeruginosa* produces two major siderophores; pyoverdine (Pvd) and pyochelin (Pch) **(Schalk, 2008)**. Siderophores are low-molecular-weight chelating agents (200–2000 Dalton), characterized by an extremely high affinity for iron. They are able to deliver iron into bacteria via active transport systems **(Boukhalfa and Crumbliss, 2002; Winkelmann, 2002)**.

Pyoverdine is a peptidic siderophore containing two hydroxamic groups and a fluorescent dihydroxyquinoline chromophore, which create a very efficient iron chelation center **(Visca *et al.*, 2007)**, while pyochelin is a salicylate-based siderophore with a lower affinity for iron **(Brandel *et al.*, 2012).**

Both siderophores are actively transported across the outer membrane upon binding to specific receptors, namely, the *FptA* and *FpvA* outer membrane proteins for pyochelin and pyoverdine transport, respectively **(Schalk and Guillon, 2013)**.

Six genes encode the proteins required for pyoverdine synthesis have been identified in *Pseudomonas aeruginosa* PAO1 strain. Expression of these six genes requires a sigma factor *pvdS* **(Lamont and Martin, 2003)**.

The *pvcABCD* gene cluster from *Pseudomonas aeruginosa* has been linked to the biosynthesis of the pyoverdine chromophore. In addition, the *pvc* gene cluster is positively regulated by *PtxR*, a LysR transcription factor **(Clarke-Pearson and Brady, 2008).**

Furtherly, the *pchDCBA* and *pchEF* genes responsible for biosynthesis of the pyochelin siderophore and its precursors salicylate and dihydroaeruginoate (Dha) are clustered with the *pchR* regulatory gene on the chromosome of *Pseudomonas aeruginosa*. The 4.6-kb region located downstream of the *pchEF* genes was found to contain three contiguous genes, *pchG*, *pchH*, and *pchI*, probably forming a *pchEFGHI* operon. The occurrence of mutation in *pchG* would abolish pyochelin formation, whereas mutations in *pchH* and *pchI* did not affect the amounts of salicylate, Dha, and pyochelin produced. So *pchG* is essential for pyochelin formation in *Ps. aeruginosa* **(Reimmann *et al.*, 2001).**

The current study aims to detect siderophores produced by *Ps. aeruginosa* phenotypically and genotypically.

## MATERIALS and METHODS

### Bacterial strains

A total of 120 clinical isolates of *Pseudomonas aeruginosa* were included in this study. They were recovered from 300 clinical specimens obtained from patients with wound infections, respiratory tract infections, urinary tract infections and burn infections admitted to Zagazig University Hospitals and Al-Ahrar hospital in Zagazig. A standard strain of *Ps. aeruginosa* (PAO1) was included in this study as a positive control for siderophore production.

### Identification of *Pseudomonas aeruginosa*

The clinical isolates were identified morphologically by Gram stain and biochemically according to **Koneman *et al* (2006)** including pyocyanin production and oxidase tests.

### Quantitative assay for siderophore production by Chrome azurol sulfonate (CAS) - Shuttle assay

CAS solution was prepared by adding 7.5 ml of 2 mM CAS solution to 1.5ml of iron solution (1 mM $FeCl_3.6H_20$ in 10 mM HCl) and 50 ml hexadeclytrimethylammonium bromide (HDTMA) solution. CAS solution was mixed with 30 ml piperazine-N,N-bis(2-ethanesulfonic acid) (PIPES) buffer and the volume was brought to 100 ml by distilled water.

Bacteria were grown in a minimal medium (M9 medium) at 37˚C for 24 hours with shaking at 100 rpm. The cells were removed by centrifugation at 10000 rpm for 10 minutes at 4˚C using Hermle cooling centrifuge, Germany. Aliquots of 0.5 ml of the supernatant were mixed with aliquots of 0.5 ml of CAS solution and 10µl shuttling solution (5-sulfosalicylic acid). After few minutes the orange color developed was assessed spectrophotometrically at 630 nm by using BioTek Synergy HT microplate reader, USA. The same procedure was performed also for reference solution (minimal medium + CAS dye + shuttle solution). Percentage of siderophore was estimated using the following formula:

(Ar-As)/Ar *100 = % of siderophore

Where Ar is the Absorbance of reference (CAS assay solution + un-inoculated medium + shuttle solution) and As is the Absorbance of the sample (CAS assay solution + cell-free supernatant+ shuttle solution) **(Schwyn and Neilands, 1987; Tank *et al.*, 2012).**

### Genotypic detection of siderophores

The phenotypic detection of siderophores was confirmed genotypically among the *Ps. aeruginosa* isolates by PCR,

where the genes encode synthesis of pyoverdine and pyochelin and their receptors were investigated.

The genomic DNA (gDNA) was extracted by picking a colony from agar plate using a sterile pipette tip and resuspended into 20 µl of distilled water. The mixture was vortexed for 10 s then heated in water bath at 98 °C for 5 min. The lysate was centrifuged and the resulting supernatant was collected, diluted with distilled water at a 1:3 dilution ratio, and subjected to PCR analysis **(Reischl *et al.*, 2000)**. Each PCR mixture contained 10 µl of MyTaq$^{TM}$ master mix (Bioline Reagents Limited, UK), 1.5 µl of forward primer, 1.5 µl of reverse primer, 2 µl of gDNA template and nuclease free water to 20 µl. The primers used in this study are listed in (Table 1). Amplification reaction of each gene listed in (Table 2). The PCR was performed in Biometra T-personal thermocycler (Goettingen, Germany). The PCR products as well as Gene-Ruler 100 bp and 1Kb DNA Ladder (Thermoscientific Inc, USA) were separated on 1.5% agarose gel, stained with 2 µl of EtBr, and visualized by UV transilluminator and photographed.

**Table1. Primer sequences and amplicon sizes of target genes.**

| Target Genes | Primer sequence 5′-3' | Amplicon Size (bp) | Reference |
|---|---|---|---|
| *pvdS*-F | 5`GCAGAATTCTCCGCAGCAAGGTGATTTCCATG-3` | 573 | **(Leoni *et al.*, 1996)** |
| *pvdS*-R | 5`CGCCAAGCTTAGCGGCGGGCGCTGAGATGGGT-3` | | |
| *ptxR*-F | 5`- TCTAGACCCGTCCGGACCCACTTC-3` | 990 | **(Stintzi *et al.*, 1999)** |
| *ptxR*-R | 5`-AAGCTTGCCCAGCCTCATTCGCTCTG-3` | | |
| *fpvA*-F | 5`-GAGCTCGAAGAGCAATCA CCCAT-3` | 2448 | **(James *et al.*, 2005)** |
| *fpvA*-R | 5`-AAGCTTGGCGTTCTTTTTCGCA-3` | | |
| *PchG*-F | 5`ATGCCAGAGGAGGCGAGCATATGAGCGAC*GTT*CG | 1047 | **(Reimmann *et al.*, 2001)** |
| *PchG*R | TTCCG-3` | | |
| | 5`AGCAGGCGCCACAGCACCGCTCGAGCGAGGCTTG CTCC-3` | | |
| *fptA*-F | 5`-GTGACGAGCTCAATACGGGCCG-3` | 2163 | **(Visser *et al.*, 2004)** |
| *fptA*-R | 5`-CCCCAAGCTTGACGCCATCAGA-3` | | |

**Table 2. Amplification reaction cycles of genes**

| Thermal Step | Gene tested | | | | | Number of cycles |
|---|---|---|---|---|---|---|
| | *PvdS* | *PtxR* | *FpvA* | *PchG* | *FptA* | |
| Initial denaturation | 95°C for 3 min | 95°C for 3 min | 95°C for 3 min | 95°C for 3 min | 95°C for 3 min | 1 |
| Denaturation | 95˚C for 20 sec | 95˚ C for 30 sec | 95˚ C for 30 sec | 95˚ C for 30 sec | 95˚ C for 30 sec | |
| Annealing | 60˚C for 30 sec | 50˚C for 50 sec | 40˚C for 30 sec | 46˚C for 30 sec | 56˚C for 30 sec | 30 |
| Elongation | 74°C for 1 min | 72°C for 1 min | 72°C for 2 min | 72°C for 1 min | 72°C for 2 min | |
| Final elongation | 72°C for 5 min | 72°C for 7 min | 72°C for 7 min | 72°C for 7 min | 72°C for 7 min | 1 |

**Electrophoresis and visualization of PCR amplicons**

The running chamber was assembled on a horizontal section of the bench. Sufficient electrophoresis tris acetate EDTA buffer (1X TAE) was added to fill the electrophoresis tank and to cast the gel. The agarose solution (1.5%) was

prepared in TAE buffer (1X) and heated in a microwave oven to dissolve the agarose. When the molten gel has cooled, ethidium bromide (EtBr) was added to a final concentration of 0.2µg/ml. The gel solution was mixed thoroughly by gentle swirling. The molten agarose was poured into the running chamber with an appropriate comb and left to solidify (30-45 minutes). The comb was removed after flooding the gel with TAE buffer. The gel was mounted in the electrophoresis tank. Just enough electrophoresis buffer was added to cover the gel to a depth of 1 mm. Each DNA sample (10 µl) was loaded carefully in separate well using micropipette then was separated according to its molecular size by electrophoresis at 4v/cm. The master mix used is supplemented with two tracking dyes that allow for direct loading of the PCR product on a gel. The electrophoresis power was shut off after the tracking dyes band reached to nearly the end of the gel length. The gel was visualized on UV transilluminator and photographed **(Sambrook and Russell, 2001).**

## RESULTS

### Identification of *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* isolates were identified as Gram-negative rods. They produced pyocyanin and fluorescein on cetrimide agar and they were motile, oxidase positive and grew at 42˚C.

### Phenotypic determination of siderophore production

In order to screen for siderophore production, the method of CAS- Shuttle assay was used. The presented results were the average of siderophore percentage from 4 experiments. The tested isolates showed variable abilities in siderophore production (Table 3 and Fig1). High siderophore production was suggested (if the percentage is ≥ 60%), intermediate (if the percentage is ≤50% and ≥30% ) and low (if the percentage is ≤29%). PAO1 showed 60% siderophore production. High siderophore production was observed in 63.33% of total isolates. Intermediate production of siderophore was detected in 30% while 6.66% of total isolates showed low siderophore production.

**Table 3. Siderophore production by *Ps. aeruginosa* isolates.**

| Percentage (%) of siderophore | Number of isolates |
|---|---|
| 60% | PAO1 |
| ≥80% | 6 |
| ≤79%-70% | 43 |
| ≤69%-60% | 27 |
| ≤59%-50% | 17 |
| ≤49%-40% | 12 |
| ≤39%-30% | 7 |
| ≤29%-20% | 6 |
| ≤19%-10% | 1 |
| ≤9%-1% | 1 |

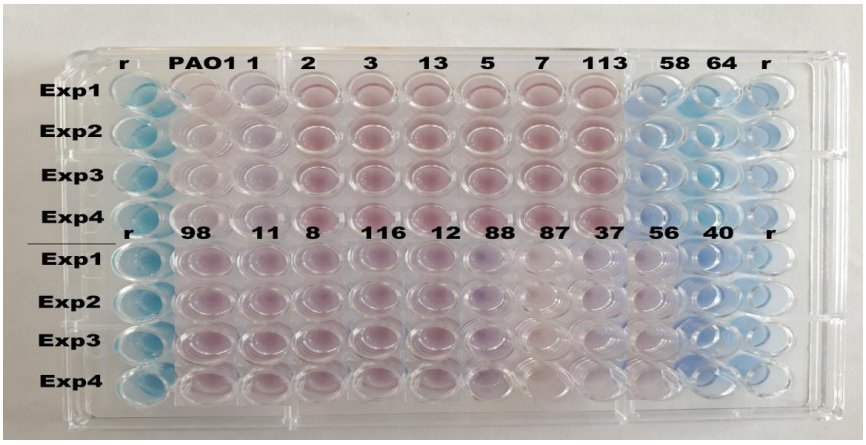


**Fig 1. siderophore detection by CAS shuttle assay reference (CAS assay solution + uninoculated medium + shuttle solution)**

**Genotypic detection of siderophores**

To investigate the presence of siderophore encoding genes of 56 isolates which they were selected to represent the different percentage levels of siderophore production.

Upon detection of pyoverdine and its receptor, it was found that PAO1 strain and all clinical isolates gave a single band at 573 bp match to *PvdS* gene (Figure 2-A). For *PtxR* gene, PAO1 strain and all clinical isolate gave a single band at 990 bp (Figure 2-B). For amplification of *fpvA* gene encoding outermembrane receptor, PAOI strain and all clinical isolate gave single band at 2448 bp (Figure 2-C).

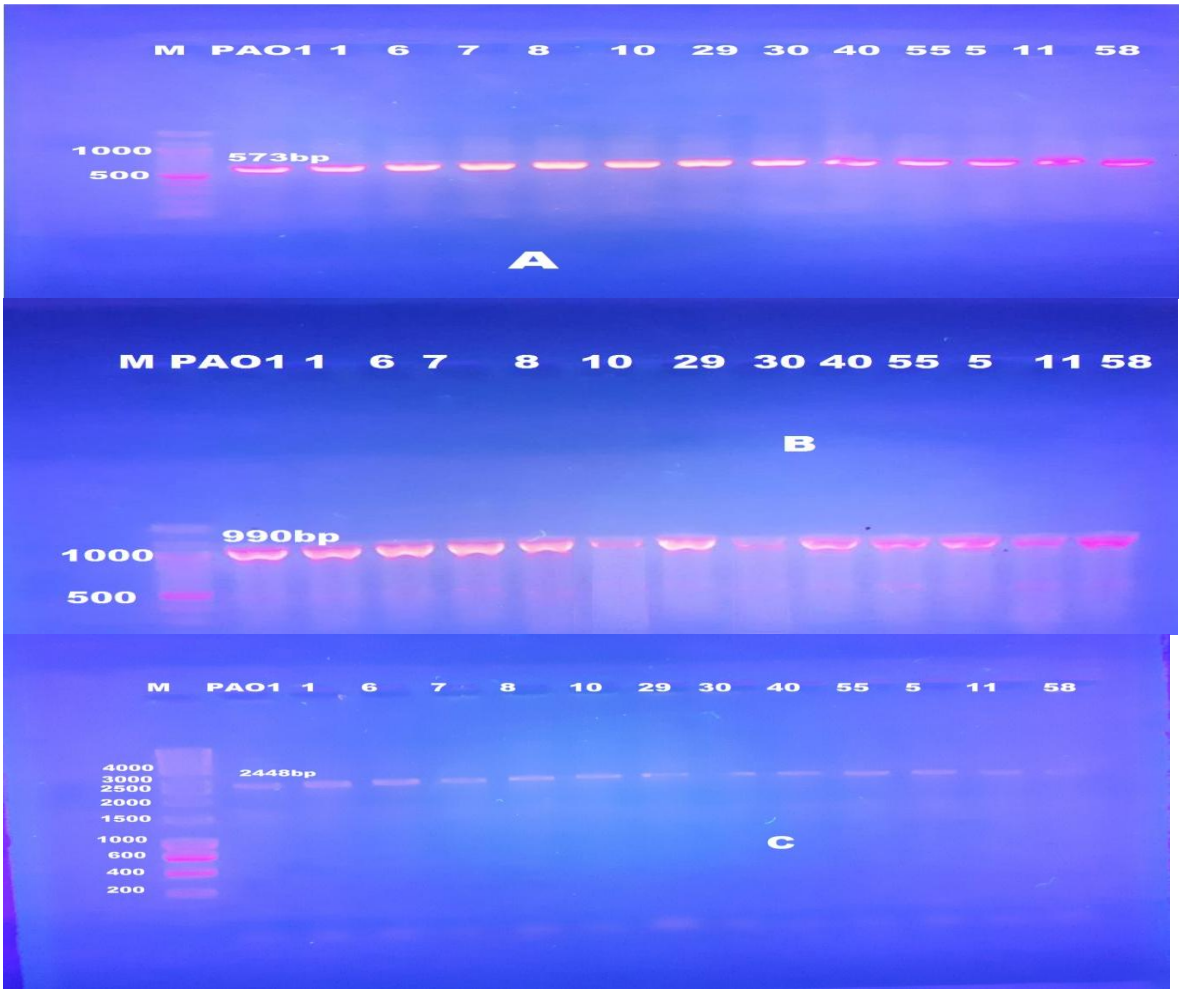


**Fig2. Gel electrophoresis of PCR products for detection of pyoverdine genes and its receptor of representing isolates. Fig 2A.** detection of *PvdS* gene in all isolates gave a single band at 573bp, **Fig2B.** detection of *PtxR* gene in all isolates gave a single band at 990bp**, Fig2C.**detection of *fpvA* gene in all isolates gave a single band at 2448bp. **bp:** base pair. **M:** marker.

Upon detection of pyochelin and its receptor, it was found that a single band of expected product size of 1047bp confirmed the presence of *PchG* gene in 85.7% of isolates and in PAO1 strain (Figure 3-A). In case of *fptA* receptor gene encoding outermembrane receptor, PAO1 strain and 85.7% of isolates gave a single band at 2163bp (Figure 3-B).

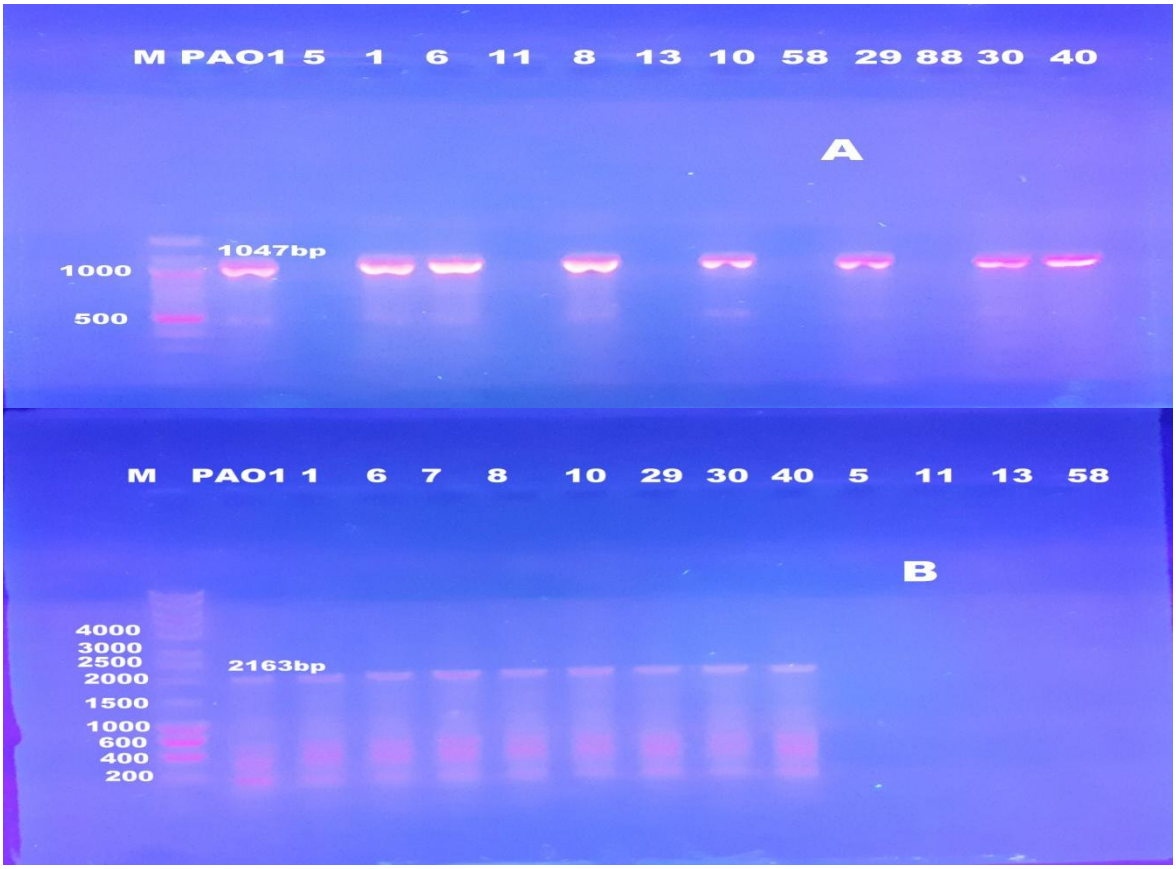


**Fig 3. Gel electrophoresis of PCR products for detection of pyochelin gene and its receptor. Fig 3A.** detection of *PchG* gene in PAO1 and clinical isolates gave a single band at 1047bp, **Fig3B.** detection of *fptA* gene in PAO1 and clinical isolates gave a single band at 2163bp**.**

## DISCUSSION

*Ps. aeruginosa* is a Gram-negative bacteria frequently found in soil, marine habitats, plants, animals, and humans **(Filiatrault *et al.*, 2006)**. It is responsible for 10–15% of the nosocomial infections worldwide **(Strateva and Yordanov, 2009)**.

This study was performed to detect and quantify siderophores produced by *Ps. aeruginosa* isolates. Moreover, for genotypic investigation of pyoverdine and pyochelin, the genes amplified by PCR were detected.

The CAS assay is the universal assay for siderophore detection and is based on a siderophore's high affinity for ferric iron **(Louden *et al.*, 2011).** In this study, *Ps. aeruginosa* isolates showed variable siderophore production by CAS-Shuttle assay. The production of sideophore was detected by the change in the colour of CAS from blue to orange (yellow brown). When comparing the average percentage of siderophore production between isolates according to their sources, the maximum production is 72.17% were found in isolates from urinary tract infections, followed by those recovered from respiratory tract infections where the production was 71.45 %, followed by wound infections isolates where the production was 55.48% and the least siderophore level was found in burn infections isolates where average percentage of production was 52.20%. These results were in consistent with that reported by **Ali and Vidhale (2011)** who estimated the amount of siderophore produced by different *Ps. aeruginosa* isolates. They found that amount of siderophore production varied in clinical isolates and the maximum siderophore

production was found in strains isolated from urinary tract infections, followed by wound and burn infection, respectively.

The presence of *PvdS*, *PtxR* and *fpvA* genes in all clinical isolates confirmed their importance in pyoverdine and its receptor formation in *Pseudomonas aeruginosa* (Lamont and Martin, 2003; Stintzi *et al.*, 1999). For pyochelin production, detection of *PchG* and *fptA* genes were selected for investigatin because they are essential for pyochelin synthesis and its specific receptor **(Reimmann *et al.*, 2001; Visser *et al.*, 2004).**

Our data show that pyoverdine genes were detected in all clinical isolates, while pyochelin genes were detected in only 85.7% of isolates, moreover, some of isolates which have pyoverdine genes and lack pyochelin genes showed high siderophore production. Collectively, the above findings may explain that pyoverdine has the main role in iron chelation while pyochelin has a secondary role in iron uptake.

**Conclusion**

Siderophores have a vital role in iron uptake in *Ps. aeruginosa*. Pyoverdine has a higher affinity for iron than pyochelin confirmed by phenotypic and genotypic detection.

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Zagazig J. Pharm. Sci. June, 2018 ISSN 1110-5089
Vol. 27, Issue 1, pp. 22- 30 ISSN (on-line) 2356_9786

# الكشف عن السيداروفورات من عزلات السودوموناس اريجينوزا بواسطة الطرق النمطية و الجينيه.

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السودوموناس اريجينوزا بكتيريا تسبب العديد من الأمراض المعديه للانسان مثل المسالك البولية ، والجهاز التنفسي ، والعدوي المصاحبه للحروق بالإضافة إلى تسمم الدم. السيداروفورات (ناقلات الحديد) هي عوامل مخلبية للحديد التي تنتجها البكتيريا. الحديد هو عنصر أساسي لنمو معظم الكائنات الحية الدقيقة. السودوموناس اريجينوزا بكتيريا تنتج نوعين من السيداروفورات الرئيسية البيوفردين و البيوكلين.

تم التعرف علي عزلات السودوموناس اريجينوزا شكليا ، وباختبارات الكيمياء الحيوية. تم قياس إنتاج السيداروفورات بواسطة مقايس سلفونات الكروم (CAS) والكشف الجيني عن طريق تفاعل البلمرة المتسلسل.

تباينت إنتاج سيدروفورات بين العزلات السريرية. تم العثور على إنتاج عالي في ٦٣,٣٣ ٪ من العزلات. وتم الكشف عن إنتاج وسيط من سيدروفورات في ٣٠ ٪ ، في حين أظهر ٦,٦٦ ٪ من العزلات مستوى منخفض من سيدروفورات. ولقد اوضح الكشف الجيني للجينات المسؤولة عن تكوين ناقلات الحديد ومستقبلاتها عن وجود جينات *PvdS* و *PtxR* و *FpvA* المسئولة عن تكوين البيوفردين و مستقبلاته في جميع العزلات. في حين تم الكشف عن وجود جيني *pchG* و *FptA* المسئولين عن تخليق البيوكلين و مستقبلاته في ٨٥,٧ ٪ من العزلات.

السيداروفورات هي ناقلات للحديد مهمة في السودوموناس اريجينوزا بكتيريا و تم استنتاج ارتباط كبير بين التحليلات الوراثية والنمطيه في إنتاج السيداروفورات.