Detection of Quorum Sensing Genes of Pseudomonas aeruginosa Isolated from Different Areas in Iraq

Huda Abbas Mohammed¹, Ayaid Khadem Zgair²*

¹Environmental Research and Studies Center, Babylon University, Babylon, Iraq
²Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen. Quorum sensing (QS) is one of processes that are responsible for biofilm formation. P. aeruginosa can live in different environments, some of which are pathogenic (clinical isolates) and some that are found outside the body (environmental isolates). The present study aimed to determine the presence of a number of genes responsible for QS in clinical and environmental isolates of P. aeruginosa. In the present study full DNA was separated from all environmental and clinical isolates that contained seven genes (rhlA, rhlR, rhlI, lasR, lasI, lasB, phzA1) associated with QS occurrence. The total DNA was separated from all environmental and clinical isolates (PAE1, PAE2, PAE3, PAE4, PAE5, PAE6, PAE7, PAE8, PAE10, PAE11, PAE12, PAE14, PAC1, PAC2, PAC5, PAC7, PAC8, PAC9 and PAC10). This study found that all studied environmental and clinical isolates contained the seven genes rhlA, rhlR, rhlI, lasR, lasI, lasB, phzA1, which was associated with QS occurrence.

Keywords: Pseudomonas aeruginosa, biofilm formation, PCR, quorum sensing, DNA extraction.

الكشف عن جينات استشعار النصاب في Pseudomonas aeruginosa المعزولة من مناطق مختلفة في العراق

هدى عباس محمد1 أياد كاظم زغير²*

1 مركز البحوث والدراسات البيئية، جامعة بابل، بابل، العراق
2 كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة

إن بكتريا الزائفة الزنجارية هي من مسببات الأمراض الانتهازية. يعتبر استشعار النصاب (QS) أحد العمليات المسؤولة عن تكوين الأغشية الحيوية الرقيقة حيث يمكنها من العيش في بيئات مختلفة، وبعضها مرض (عزلات سريرية) وبعضها موجود خارج الجسم (عزلات بيئية). تهدف الدراسة الحالية إلى تحديد وجود P. aeruginosa عدد من الجينات المسؤولة عن QS في العزلات السريرية والبيئية للكثيرها، في هذه الدراسة تم فصل كل الحمض النووي عن جميع العزلات البيئية والسريرية التي تحتوي على سبعة جينات تشمل rhlA، وتصلح كل الحمض النووي، phzA1, lasB, lasI, lasR, rhlI, rhlR، والتي ارتبطت بحدوث QS. تم فصل الحمض النووي الكلي عن جميع العزلات البيئية والسريرية (PAE1, PAE2, PAE3, PAE4, PAE5, PAC1, PAC2, PAC5, PAC7, PAC8, PAC9 and PAC10).

*Email: ayaid.zgair@sc.uobaghdad.edu.iq
**Introduction**

*P. aeruginosa* is a stick-shaped gram-negative pathogen that is associated with serious illnesses and infections such as pneumonia and various sepsis syndromes in humans, as well as it causes lung cancer. In addition, *P. aeruginosa* isolates infect immune compromised people, burns patients and individuals suffering from cystic fibrosis [1]. Its infection treatment is a big challenge as this bacteria is highly resistant to a wide spectrum of antibiotics [2]. *P. aeruginosa* swarming requires releasing of two external factors, rhamnolipids and 3-(3-hydroxyalkanoyloxy alkanoic acids) that play role as wetting factors [3]. However, a tiny chemical molecule called auto-inducer has the main role in connecting and leading the bacterial cell to combine and form a biofilm [4]. There is a correlation between the ability of bacteria to form biofilm and its resistance to antibiotics [2]. Previous studies have showed that environmental isolates of *P. aeruginosa* have an ability to form biofilm [5]. Quorum sensing is a process of gene organizing that uses the chemical indicators by many hosts-related bacteria for observing accumulation of population of bacteria in a certain area [6]. It is well known that the quorum sensing style differs from bacterium to bacterium, i.e., gram negative bacteria use acyl-homoserine lactone (AHL) [7]. The adhesion part of biofilm formation and quorum sensing regulation to synthesis the signal such as *lasI* in *Pseudomonas* bacteria which have role to synthesis quorum sensing signal regulates by many genes [8]. In biofilms, the gene expression depends on bacterial response to the native ecological conditions.

Over the last decade, several researchers studied AHL indicators that are used by gram negative bacteria in the quorum sensing. The results clarified that *P. aeruginosa* genome expression of extracellular virulence agents created by *P. aeruginosa* is organized through quorum sensing [9]. Other studies found that quorum sensing has two specific patterns in *P. aeruginosa* of the *lasI* gene in the *las* quorum sensing patterns that are responsible for the diffusible extracellular indicator, N-(3-oxododecanoyl)-L-HSL. The first pattern is a *las* system, while the second pattern is *rhl* system that reacts together with *LasR* [10] to operate virulence genes including *lasB*, *lasA*, *apr*, *toxA* and *lasI* [11]. Composition of the siderophore pyoverdine also is stimulate by the *las* system[12]. The 3OC12-AHL seems to division into cell membranes, and *P. aeruginosa* efflux pumps support divide the movement of this signal to the external environment although 3OC12-HSL is diffusible . The synthesis of N-butryryl-L-HSL (C4-AHL) catalyzes by *rhl* product[13]. Pyocyanin, Cyanide, and Chitinase are virulence factors, they are positively organized by the *rhl* system[14].

Previous studies highlighted the role of several genes in QS directly and indirectly such as *las* and *rhl* [15]. These systems are coiled in a hierarchical manner, *las* system controls *rhl* system together, the transcriptional and posttranslational fields, It also has a role in the production and development of biofilms. In addition to antibiotic resistance[16]. A complicate phenazin biosynthetic lane found in *P. aeruginosa*, that consist of two symmetric core loci included *phzA1B1C1D1E1F1G1*, *phzA2B2C2D2E2F2G2*, they are responsible for the production of phenazine-1-carboxylic acid in addition to other three supplemental genes inclusive *phzM*, *phzS* and *phzH* encoding individual enzymes implicated in the transformation of phenazine-1-carboxylic acid to pyocyanin,1-hydroxyphenazine, in addition to phenazine-1-carboxamide [17]. The genes that are responsible for QS had been covered by several studies but the question raised here is regarding the existence of QS genes in clinical isolates of *P. aeruginosa* in parallel with QS genes that are present in environmental isolates of *P.
The current study provided an answer to this question

**Materials and Methods**

**Samples Collection**

Fifty-three samples were collected from burn wound, sputum of patients suffering from respiratory tract infections and ear infections. Samples were collected from indoor patients after getting an ethical approval from the Ethical Committee in the Department of Biology, College of Science, University of Baghdad and signed consents from the patients. Samples were cultured onto nutrient agar under sterile conditions. Subsequently, seventy samples collected from Shatt Al-Hillah and the Al-Yahudia rivers, were placed in sterile glass containers and 0.1 ml from liquid specimens were cultured onto nutrient agar [18].

**P. aeruginosa Isolation and Identification**

Suspicious isolates of *P. aeruginosa* were cultured onto cetrimide agar. The isolated colonies of suspected *P. aeruginosa* appeared yellow-green and blue-green under fluorescent down ultra violet rays light. Standard method was followed to identify bacterial isolates. VITEK 2 DensiCheck tool (bioMe´rieux) (ID-GNB card) was used in the present study [19, 20].

**QS genes Detection**

Nucleic acid was extracted to detect several genes (*rhlA*, *rhlR*, *rhlI*, *lasR*, *lasI*, *lasB*, and *phzA1*) that are responsible for QS and biofilm formation in the clinical and environmental isolates of *P. aeruginosa*. DNA was extracted according to the manufacturer's instructions of G-spin™iNtRON kit. Primers were used for the detection of genes by polymerase chain reaction (PCR). Sequences of primers used in the current study are mentioned in Table 1.

| Table 1: Description of primers used in PCR technology to detect some of the studied QS and biofilm genes [21] |
|---|---|---|---|---|---|
| Amplicon | Primer | Sequence 5'-3' | Position | Length (bp) |
| rhlA | rt_rhlA-F | AACATTCAACGTGGTGCTG | 155 | 100 |
| | rt_rhlA-R | ATTTCCACCTCGTCTCCTT | 254 |  |
| rhlR | rt_rhlR-F2 | CTGGGCTTCATTACTACGC | 112 | 124 |
| | rt_rhlR-R2 | CCCGTTAGTTCTGACATCTGTT | 215 |  |
| rhlI | rt_rhlI-F | GGAGCGCTATTTCGTTG | 429 | 100 |
| | rt_rhlI-R | GTAGGCCGGGAAGCTGAT | 528 |  |
| lasR | rt_lasR –F | CGGTTTTCCTAGGCTGGAAC | 15 | 100 |
| | rt_lasR – R | GCCGAACAGGATCTTCGAG | 114 |  |
| lasI | rt_lasI –F | GGCTGGACGTAGTGTCTGG | 94 | 100 |
| | rt_lasI – R | CCTGGGCTTCAGGAGCTG | 193 |  |
| lasB | rt_lasB –F | AACCCTGCGTCTACCTGTT | 1279 | 100 |
| | rt_lasB –R | TGGTGCGGTCCGCTGTA | 1378 |  |
| phzA1 | rt_phzA1 –F | AACCCTACATCCATTCCCTG | 355 | 100 |
| | rt_phzA1 – R | CGGCTATTCCCAATGC | 454 |  |
Two microliter of extracted DNA (extracted from all clinical and environmental isolates of P. aeruginosa) was mixed with 8 μl of the master mix (BioNeers, USA), 7.5 μl of free water ddH₂O and 0.5 μl of MgCl₂. After adding 2μl of primer to the final mix and it was then placed in PCR machine (Biometra, Germany) for incubation at 94°C for 5 min. The temperatures and duration of each cycle (35 cycles) is shown in Table 2.

Table 2: PCR reaction schedule for 35 cycles that were applied for detecting rhlA, rhlR, rhlI, lasR, lasI, lasB, and phzA1 in the clinical and environmental isolates of P. aeruginosa.

| Stage                | Temperature (°C) | Time     | Number of Cycle |
|----------------------|------------------|----------|-----------------|
| Initial denaturation | 94               | 5 min    | 1               |
| Denaturation         | 94               | 30 sec   | 35              |
| Annealing            | 55               | 30 sec   |                 |
| Elongation           | 72               | 30 sec   |                 |
| Final elongation     | 72               | 5 min    | 1               |

Gel Electrophoresis

0.8 gram of the agarose was added to 80 ml of the Tris-borate-EDTA (TBE) buffer. The mixture was heated using microwave oven. Subsequently, the mixture temperature was decreased to 50-60°C. Finally, 4 μl of Red Safe stain (iNtRON, Korea) was added to agarose prior to solidifying. Comb was put at one end of the gel template and then the agarose mixture was poured into template. Next it was left at room temperature for 30 minutes. After that the tank tray was loaded with TBE buffer. Finally, after releasing the comb from the template, the template was placed into the tank tray. DNA samples were prepared by mixing 1 μl of bacterial DNA by blending 1 μl of dye. The samples and leader were loaded in the agarose wells and then the electrophoresis device (BIORAD, UK) was turned on at 75 volt/cm, 20 mA. Gel documentation system was used to get the pictures of bands [22].

Results and Discussion

In the current study, a number of clinical samples were collected from patients suffering from burns wound infection, respiratory tract infections, otitis and a number of river water samples. A number of P. aeruginosa isolates were separated and diagnosed based on some chemical tests and the colony shapes on the culture media, where 10 isolates (PAC1, PAC2, PAC3, PAC4, PAC5, PAC6, PAC7, PAC8, PAC9 and PAC10) were pre-identified as P. aeruginosa isolated from clinical samples and 12 isolates (PAE1, PAE2, PAE3, PAE4, PAE5, PAE6, PAE7, PAE8, PAE9, PAE10, PAE11 and PAE12) were pre-identified as P. aeruginosa isolated from river water. In order to obtain a more accurate diagnosis, all isolates were re-diagnosed using the VITEK 2 DensiCheck technique. From the 10 clinical isolates only 7 were confirmed as P. aeruginosa (PAC1; PAC2; PAC5; PAC7; PAC8; PAC9; and PAC10) and from the 12 environmental isolates only 11 (PAE1; PAE2; PAE3; PAE4; PAE5; PAE6; PAE7; PAE8; PAE10; PAE11; PAE12) were confirmed as P. aeruginosa.

In the current study, DNA was extracted from both clinical and environmental isolates of P. aeruginosa. PCR technique was applied to determine the presence of seven genes (rhlA, rhlR, rhlI, lasR, lasI, lasB, and phzA1) associated with the QS and biofilm phenomenon.
results showed that the studied genes were found in all clinical and environmental isolates of *P. aeruginosa*.

Figures 1-7 show an electrophoresis image from PCR results that were taken by gel documentation system which showed the amplification genes by PCR technology. The figures show that all the identified genes had the number of base pairs (bp) around 100, except *rhIR* gene where the number of base pairs was 120.

**Figure 1:** Agarose gel electrophoresis photograph for *rhlA* amplified by PCR technology. The number of base pairs was 100. The samples were run at 75 volt and 20 mA for 1.5 hours, the concentration of agarose was 10 gm/l.

**Figure 2:** Agarose gel electrophoresis photograph for *rhlR* amplified by PCR technology. The number of base pairs was 120. The samples were run at 75 volt and 20 mA for 1.5 hours, the concentration of agarose was 10 gm/l.
Figure 3: Agarose gel electrophoresis photograph for rhlI amplified by PCR technology. The number of base pairs was 100. The samples were run at 75 volt and 20 mA for 1.5 hours, the concentration of agarose was 10 gm/l.

Figure 4: Agarose gel electrophoresis photograph for lasR amplified by PCR technology. The number of base pairs was 100. The samples were run at 75 volt and 20 mA for 1.5 hours, the concentration of agarose was 10 gm/l.

Figure 5: Agarose gel electrophoresis photograph for lasI amplified by PCR technology. The number of base pairs was 100. The samples were run at 75 volt and 20 mA for 1.5 hours, the concentration of agarose was 10 gm/l.
Figure 6: Agarose gel electrophoresis photograph for lasB amplified by PCR technology. The number of base pairs was 100. The samples were run at 75 volt and 20 mA for 1.5 hours, the concentration of agarose was 10 gm/l.

Figure 7: Agarose gel electrophoresis photograph for lasB amplified by PCR technology. The number of base pairs was 100. The samples were run at 75 volt and 20 mA for 1.5 hours, the concentration of agarose was 10 gm/l.

Several previous results showed the ability of *P. aeruginosa* to form biofilm [23, 24]. There are different factors that play an important role in its formation, QS phenomenon being one of them [25]. There are two pyramidal structures of the QS for *P. aeruginosa*, las and the rhl (26). The condensation of molecule signaling for 3-oxo-dodecanoyl of the homoserine lactone increased when the inhabitant’s thickness grew in particular to form a complex with lasR. This complex operates the genes transcription as a lasI, rhlR and other genes [26].

rhl structure consists of the rhlR protein and the auto inducer synthesize RhlII which is incorporated to form auto inducer N-butryl homoserine lactone. These structures are twisted in the manner of pyramidal structures. Indeed, the las structure is controlled by the rhl structure, in terms of transcriptional and also the next translational areas together. According to this fact, the condensation of marking molecule 3-oxo-dodecanoyl homoserine lactone which interacts with LasR at to activate a number of virulence genes such as lasB. These structures are wrapped up in the pyramidal manner. Our results proved that proteases, exotoxin A, rhamnolipids and pyocyanin are considered as the virulence factors in the *P. aeruginosa*. This formation and organization is considered as an indicator of the QS, and also merge the formation and development of the biofilm. It also plays an important role in antibiotic resistance [16].
The results of the present study agree with recent research that indicates the selection genes chosen as targets, and also as one of the causes of epidemic, help spread of the *P. aeruginosa* bacteria. Further, the clinical isolates were able to form biofilm which had *lasR*, *rhlI*, and *rhlR* genes. As for the general, isolates included *lasI*. This finding also agrees with a previous study [27].

**Conclusions:**
The total DNA separated from all environmental and clinical isolates (PAE1, PAE2, PAE3, PAE4, PAE5, PAE6, PAE7, PAE8, PAE10, PAE11, PAE12, PAE14, PAC1, PAC2, PAC5, PAC7, PAC8, PAC9 and PAC10) contained the following seven genes: *rhlA*, *rhlR*, *rhlI*, *lasR*, *lasI*, *lasB*, *phzA1*, which are associated with QS and biofilm formation.

**Abbreviations**

DNA: Deoxyribonucleic acid; *P. aeruginosa*: Pseudomonas aeruginosa; PAE1: *Pseudomonas aeruginosa* environmental isolate 1; PAE2: *Pseudomonas aeruginosa* environmental isolate 2; PAE3: *Pseudomonas aeruginosa* environmental isolate 3; PAE4: *Pseudomonas aeruginosa* environmental isolate 4; PAE5: *Pseudomonas aeruginosa* environmental isolate 5; PAE6: *Pseudomonas aeruginosa* environmental isolate 6; PAE7: *Pseudomonas aeruginosa* environmental isolate 7; PAE8: *Pseudomonas aeruginosa* environmental isolate 8; PAE10: *Pseudomonas aeruginosa* environmental isolate 10; PAE11: *Pseudomonas aeruginosa* environmental isolate 11; PAE12: *Pseudomonas aeruginosa* environmental isolate 14; PAC1: *Pseudomonas aeruginosa* clinical isolate 1; PAC2: *Pseudomonas aeruginosa* clinical isolate 2; PAC5: *Pseudomonas aeruginosa* clinical isolate 5; PAC7: *Pseudomonas aeruginosa* clinical isolate 7; PAC8: *Pseudomonas aeruginosa* clinical isolate 8; PAC9: *Pseudomonas aeruginosa* clinical isolate 9; PAC10: *Pseudomonas aeruginosa* clinical isolate 10; PCR: poly chain reaction; AHL: acyl homoserine lactone; HSL: homoserine lactone.

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