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RESEARCH ARTICLE
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Study the Molecular Markers of Quorum Sensing in *Pseudomonas aeruginosa* Isolated from Different Clinical Infections

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ABSTRACT

The study included the collection of 100 samples were collected from different clinical sites. Clinical samples collected from patients who were admitted and visited Al- Imam Al-Sadiq Hospital in Al-Hilla city, at the period from September to November (2021). Twenty isolates were showed positive and identified as Pseudomonas aeruginosa by using selective media, biochemical tests and Vitek 2 system. Quorum sensing genes were detected in all P. aeruginosa isolates, the results showed that, LasI, LasR, rhlI and RhlR were 100% gave positive results to these genes, these results achieved using specific genes primers, which gave molecular length 295 bp, 130 bp, 155 bp and 133bp respectively.

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INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative opportunistic bacterium that lives in soil, water, and even in environments like hot tubs. A common bacterium can cause disease in mammalians. It is found in soil, water, skin flora, and most man-made environments throughout the world (Crone *et al.*, 2020). *P. aeruginosa* is a non-fermentative, aerobic Gram-negative rod, measuring (0.5) to (0.8) µm by (1.5) to (3.0) µm, almost all strains are motile by means of a single polar flagellum. It normally lives in moist environments, and uses a wide range of organic compounds for growth, thus giving it an exceptional ability to colonize ecological niches where nutrients are limited, from water and soil to plant and animal tissues (Galdino *et al.*, 2017). Infection caused by *P. aeruginosa* is common, with the burden of infections in hospitalized patients. The National Nosocomial Infections Surveillance (NNIS) system reports *P. aeruginosa* to be the second most common organism isolated in nosocomial pneumonia (17% of cases), the third most common organism isolated in both urinary tract infection (UTI) and surgical site infections (11% of cases), and the fifth most common organism isolated from all sites of nosocomial infections (9% of cases) (Restrepo *et al.*, 2019). *P. aeruginosa* cells communicate through quorum sensing (QS) system, i.e. by synthesizing small signal molecules, which depending on the density of the population correlate the regulation of virulence factors expression, biofilm development, production of secondary metabolites and interaction with hosts (Ahmed *et al.*, 2019).

P. aeruginosa employs three major interconnected QS systems that function independently and dependently involving *las*, *rhl*, *pqs* pathways as well as novel candidate *iqs* pathway regulated by several QS signal molecules. *N*-acyl homoserine lactones (AHLs) are the best characterized QS signal molecules. Different AHLs possess a homoserine lactone ring with an attached fatty acyl side chain of 4 to 20 carbons (Malešević *et al.*, 2019). Disruption of QS achieved by interference with QS signaling or interception of signal molecules is considered a key point for development of antibacterial and anti-disease strategies targeting pathogens like *P. aeruginosa* in medicine. Indeed, interference with QS signaling by QS-inhibitors (QSI) or interception of signal molecules by quorum quenching enzymes (QQE) results in a reduction of virulence regulated by QS (Grandclément *et al.*, 2016). It is considered that, QSI is a natural mechanism first developed either by QS-emitting organisms for the recycling or clearing of their own QS signals or by QSI organisms in the context of a competitive relationship with QS-signal-emitting organisms. Thus, bacteria that share ecological niche with *P. aeruginosa* during infections could be considered as promising producers of novel QSI molecules (Malešević *et al.*, 2019). The processes is controlled by QS, which include bioluminescence, sporulation, competence, antibiotic production,

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biofilm formation, and virulence factor secretion (Kalia *et al*., 2015). Gram-negative bacteria typically use *LuxI*/*LuxR*-type QS systems for controlling QS. In these systems, the *LuxI* homolog is an auto inducer synthase that catalyzes a reaction between S-adenocylmethionine (SAM) and an acyl carrier protein (ACP) to produce a freely diffusible acyl homoserine lactone (AHL) (Zhang *et al.,* 2021).

AIM OF STUDY

The aim of this study to screen of some important quorum sensing genes among *pseudomonas aeruginosa* isolates from clinical infections.

MATERIALS AND METHODS

Study Design:

This study were included 100 patients, the specimens were collected from different sites of infections (wounds, urine, sputum and Otitis media) from patients who were admitted and visited Al- Imam Al-Sadiq Hospital in Al-Hilla city, during a period of three months from September to November 2021.

Ethical Approval

All subjects involved in this work were informed and the agreement required for doing the experiments and publication of this work was obtained from each one prior the collection of samples. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee (at College of Medicine University of Babylon).

Clinical specimens

The urine, sputum, wound and otitis media specimens were collected in proper ways to avoid any possible contamination (Collee *et al*., 1996).

Identification of bacteria

Colonial morphology and microscopic examination:

Depending on its morphological properties (colony form, size , color, borders, and texture), a single colony from each primary positive culture on blood, MacConkey and nutrient agar and classify it and examine it by light microscope after being stained with Gram's stain. Biochemical tests were performed on each isolate after inspection to complete the final identification according to (Baron *et al*, 1994; McFadden, 2000) and it used the vitek2 method for *P. aeruginosa* identification.

Identification of bacterial isolates with Vitek2 System

Vitek 2 clinical microbiology used as an automatic identification (ID) instrument device.

DNA extraction

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company Geneaid, (Korea).

Detection of some of P. aeruginosa quorum sensing genes

DNA (extract from bacterial cells) was used as a template in specific PCRs for the detection of some of *P. aeruginosa* quorum sensing genes. DNA was purified from bacterial cells by using the Geneaid DNA extraction Kit. The primers used for the amplification of a fragment gene were listed in Table (1). (Al-Kilabi *et al*., 2020).

RESULTS AND DISCUSSION

Isolation of pathogenic bacteria

A total of 100 samples were collected from patients suffering from urinary tract infections were admitted and visited Al-Imam Al-Sadiq Hospital in Al-Hilla city, during a period of three months from September to November 2021. Twenty isolates were showed positive and identified as ***P. aeruginosa*** by using selective media, biochemical test system and Vitek 2 system.

Table 1: The primers, sequences, and PCR conditions

Gene name	*Primer sequence (5´- 3´)*	*Size of Bp*	*Conditions*
Las I	F: 5'-CGTGCTCAAGTGTTCAAGG-3' R:5'-TACAGTCGGAAAAGCCCAG-3'	295	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 55.7ºC, 30 sec. Step 4: 73ºC, 30.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 73ºC, 5 min. Step 7: 4ºC
Las R	F: 5'-AAGTGGAAAATTGGAGTGGAG-3' R: 5'-GTAGTTGCCGACGACGATGAAG-3	130	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 55.7ºC, 30 sec. Step 4: 73ºC, 30.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 73ºC, 5 min. Step 7: 4ºC
rhlI	F: 5'-TTCATCCTCCTTTAGTCTTCCC-3' R: 5'-TTCCAGCGATTCAGAGAGC-3'	155	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 55.7ºC, 30 sec. Step 4: 73ºC, 30.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 73ºC, 5 min. Step 7: 4ºC

Gene name	*Primer sequence (5´- 3´)*	*Size of Bp*	*Conditions*
rhlR	F: 5'-TGCATTTTATCGATCAGGGC-3' R: 5'-CACTTCCTTTTCCAGGACG-3	133	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 55.7ºC, 30 sec. Step 4: 73ºC, 30.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 73ºC, 5 min. Step 7: 4ºC

Molecular detection of quorum sensing genes in *P. aeruginosa*

The genomic *LasI* was detected in all *P. aeruginosa* isolates (100%) gave positive results to this gene, these results achieved using specific *LasI* gene primers. which gave molecular length (295 bp), the results were shown in Figure (1). In addition, molecular detection of *LasR* gene was done for all (20) *P. aeruginosa* isolates and the results showed that all these isolates (100%) isolates have this gene. The positive results for *LasR* gene were detected by the presence of (130 bp) band compared with allelic ladder as shown in Figure (2). However, molecular detection of *rhlI* gene was done for isolates that previously detected as *P. aeruginosa.* The results showed that all isolates (100%) gave positive results for this virulence gene. Positive results were detected by the presence of (155 bp) bands when compared with allelic ladder as shown Figure (3). *RhlR* gene produce transcriptional regulatory proteins that activate target genes by using PCR technique with specific forward and reverse primers. According to the results as shown in Figure (4) of the present study, it was appear that *RhlR* gene represented in 20(100%) bacterial isolated. Positive results were detected by the presence of (133bp) bands when compared with allelic ladder.

These results were agreement with results obtained by Lahij *et al.,* (2021) who found that, molecular detection of (QS) genes by used specific primers for *lasI* gene showed that (94.1%) of isolates were positive for this gene in *P. aeruginosa*. While Fattouh *et al*., (2018) found that (86.2%) of *P. aeruginosa* isolates have *lasI* gene. The *lasI* gene plays an important role in maintenance of *P. aeruginosa* biofilm, where that the signaling 3-oxo-C12-HSL (synthesized by *LasI*) is necessary for the establishment of *P. aeruginosa* biofilm, whereas a *lasI* mutant forms a flat and thin biofilm, and *lasI* is expressed in a large number of cells during the initial stage of biofilm formation (Zhou *et al*., 2018). Elnegery *et al*., (2021) found that, QS genes regulate the production of pyocyanin pigment, whereas results of pyocyanin production showed that 95% of isolates which are able to produce of pyocyanin were positive to *rhlI* gene. The important role of QS systems in pathogenesis of *P. aeruginosa* bacteria and also indicated that *P. aeruginosa* able to causing clinical infections in humans despite a weakness of QS system in some isolates. On the other hand these results was contradict with theory that QS system plays a main role in *P. aeruginosa* pathogenicity, and all virulence factor controlled by QS (Diggle & Whiteley, 2020). Li *et al*., (2018) found that, most of *P. aeruginosa* isolates were positive for QS genes, also QS plays an important role in the pathogenesis of *P. aeruginosa* infection. The *lasI* gene has important role in the production of biofilm. El-Mahdy & El-Kannishy, (2019) identified one QS deficient clinical isolate which lost all virulence factors tested, yet still caused a wound infection. Mould *et al*., (2020) identified three *P. aeruginosa*, which were defective in the production of both signaling molecules and extracellular virulence factors. Results of these two studies suggested that besides known virulence factors, there may be additional factors yet uncharacterized involved in the pathogenesis of *P. aeruginosa*.

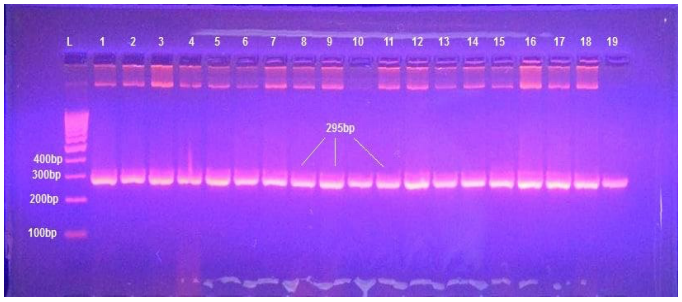

Fig. 1: Ethidium bromide stained agarose gel showing PCR amplification products with LasI gene (295bp) primers for *P. aeruginosa* extracted DNA L: ladder, (1, 2, 3,……19) samples of *P. aeruginosa* positive LasI gene.

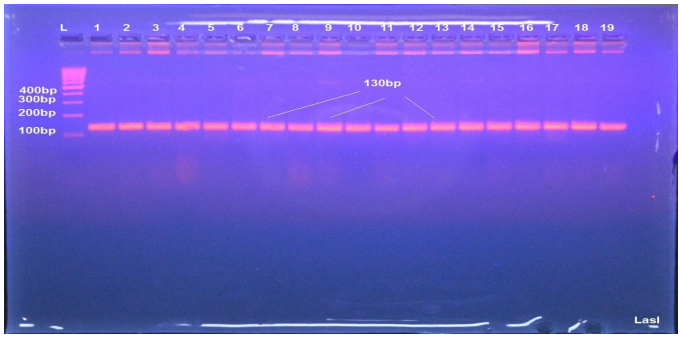

Fig. 2: Ethidium bromide stained agarose gel showing PCR amplification products with LasR gene (130bp) primers for P. aeruginosa extracted DNA L: ladder, (1, 2, 3,……19) samples of P. aeruginosa positive LasR gene.

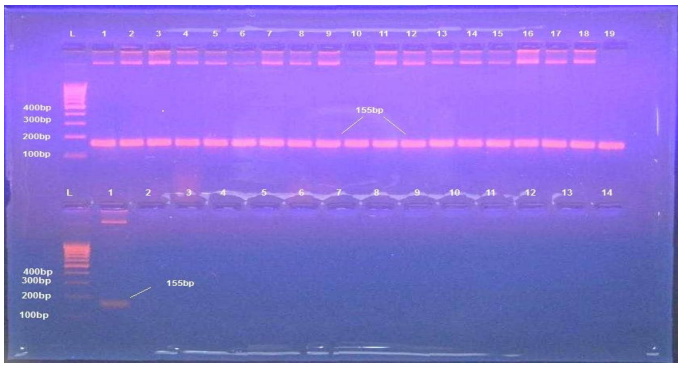

Fig. 3: Ethidium bromide stained agarose gel showing PCR amplification products with rhII gene (155bp) primers for P. aeruginosa extracted DNA L: ladder, (1, 2, 3,……20) samples of P. aeruginosa positive rhII gene.

Another possibility that may lead a QS deficient strain to cause infection is the presence of multiple *P. aeruginosa* strains in the infection site. A single patient may be infected by both QS proficient and deficient strains of *P. aeruginosa*. QS deficient strains could profit from the extracellular enzymes produced by QS proficient partners. Production of signaling molecules and/or QS-regulated factors by QS proficient strains may enable a QS deficient strain to take part in an infection

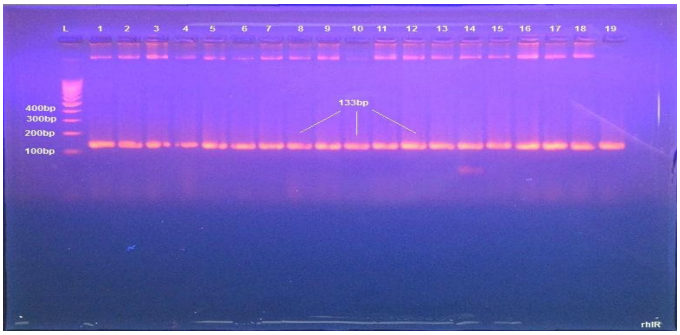

Fig. 4: Ethidium bromide stained agarose gel showing PCR amplification products with rhIR gene (133bp) primers for P. aeruginosa extracted DNA L: ladder, (1, 2, 3,.....19) samples of P. aeruginosa positive rhIR gene.

(Oh *et al*., 2017). The integration of QS into additional regulatory circuits increases the range of environmental and metabolic signals that affect QS gene expression beyond cell density as well as further tuning the timing of the QS response (Kim *et al*., 2017). Most studies on QS regulation have been done on *P. aeruginosa*, and have indicated that a large number of factors influence QS (Zhang *et al*., 2018; Yang *et al*., 2020). Biofilms play an important role in *P. aeruginosa* drug resistance. Three factors are most important to induce biofilm: quorum sensing (QS), bis-(3′-5′)-cyclic diguanosine monophosphate (c-di-GMP), and small RNAs (sRNAs). *P. aeruginosa* has its own specific QS system (PQS) besides two common QS systems, *LasI–LasR* and *RhlI–RhlR*, in bacteria. PQS is interesting not only because there is a negative regulation from RhlR to *pqsR* but also because the null mutation in PQS leads to a reduced biofilm formation (Yan & Wu, 2019). *P. aeruginosa* has three QS systems. (i) LasI–LasR that is related to the synthesis and the use of *N*-(3-oxo-dodecanoyl)-L-homoserine lactone ($3OC_{12}$-HL), whose concentration is ranged from 1 to 5 μM. (ii) RhlI-RhlR that is related to the synthesis and the use of *N*-(butyryl)-L-homoserine lactone (BHL), whose concentration is about 10 μM. (iii) *Pseudomonas* quinolone signal (PQS)-based QS, PqsABCDH-PqsR that is related to the synthesis and the use of 2-heptyl-3-hydroxy-4-quinolone (HHQ), whose concentration is about 6 μM. The first two QS systems essentially are *N*-acylated homoserine lactone (AHL)-based QS systems, and exist in many bacteria (García-Reyes *et al*., 2021). Importance of QS to establish a successful infection has been also shown in a number of different infection models such as mouse burn wound, pulmonary infection and keratitis, by employing QS deficient strains, inadequacy of QS deficient strains to establish successful infection was proposed to be associated with reduced production of virulence factors (Wille *et al*., 2020).

CONCLUSION

Quorum sensing system take a critical part in pathogenicity of *P. aeruginosa*, and it's an excellent was for bacteria to increase growth and resistant of antibiotics.

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