RESEARCH ARTICLE

Co-existence of LasI, RhI, and *Pseudomonas* Quinolone Signal Quorum-sensing Genes in Clinical *Pseudomonas aeruginosa* Isolates

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ABSTRACT

Pseudomonas aeruginosa can regulate different group actives and physiological processes through the quorum sensing mechanism. The aims of this research were to detect the presence of quorum sensing genes in 50 clinical *P. aeruginosa* isolates, which represent by (*lasI, lasR, rhlI,* and *rhlR*) and *Pseudomonas* quinolone signal (PQS) (*PgsA, PgsB, PgsC, PgsD, PgsE,* and *MvfR*) genes by Polymerase chain reaction (PCR) technique and interaction between the two systems. Isolates were subjected to test their susceptibility to 12 antimicrobial drugs, 64% of isolates showed resistance to ceftazidime, followed by carbencillin (56%), while only 8% were resistant to imipenem. In addition, all of the bacterial isolates were distributed within three multidrug-resistant (MDR) patterns, *viz.*, A, B, and C. The highest rate of MDR was showed with MDR pattern C, in which bacterial isolates showed resistance to resist (9→11) antimicrobial drugs. Results revealed that *P. aeruginosa* isolates have different gene patterns, *viz.*, A to E. According to quorum sensing genes production, pattern A found to express all the genes in LasI, RhI, and PQS system, while pattern B has a defective for the production of *lasR*, *rhlR* genes, while the same isolates have the PQS system all present. Significantly, there is a positive relationship between *las* and *rhl* system and regulation of antibiotics resistance, in which the bacterial isolates that have *las* and *rhl* genes showed high resistance to common antimicrobial agents under study. These findings suggest that PQS can function as an intercellular signal in *P. aeruginosa* that is not restricted only to alkyl homoserine lactones (AHL).

Keywords: Multidrug-resistant (MDR), *Pseudomonas aeruginosa, Pseudomonas* quinolone signal (PQS), Quorum sensing.

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INTRODUCTION

P. aeruginosa is considered one of the most gram-negative opportunistic pathogens causing a broad range of infections in healthcare settings and community.[1] *P. aeruginosa*, it stands out as a threatening and unique microorganism, as it has the ability to cause invasive disease, and to evade the immune defenses causing infections hard to be treated. Infections caused by *P. aeruginosa* are almost impossible to treat.[2] *P. aeruginosa* is one of the most problematic drug-resistant bacterium in the world today. Scientists are now facing growing clones of pan drug-resistant *P. aeruginosa*, which is responsible for moving humans to the post-antibiotic era of diseases caused by drug-resistant bacteria.[3] Multiple research reported the epidemic outbreaks caused by XDR/ MDR strains within the environment of the hospital. Concerning data have provided evidence of the existence of MDR and XDR global clones distributed in many hospitals all around the world that have been denominated high-risk clones.[4] *P. aeruginosa* is responsible for regulating different group activities and physiological processes through the quorum sensing mechanism. In the quorum sensing mechanism, bacterial cells produce, then, detect and respond to the diffusible small signal molecule.[5] Three different chemotypes of Autoinducers (AIs) have been recognized in *P. aeruginosa*: AHL used by *las* and *rhl* systems, alkyl quinolones (AQs) used by the PQS system, and 2-(2-hydroxyphenyl) thiazole-4-carbaldehyde used by the IQS system.[6] In the *las* system, N-3-oxododecanoyl-homoserine lactone, 3OC12-HSL is produced by the enzyme, which is encoded by the *lasI* gene. When *P. aeruginosa* bacteria reaches to threshold density, 3OC12-HSL will bind to LasR, which acts as a transcriptional activator; LasR, in turn, dimerizes and binds to target promoters to control gene expression of many virulence genes, while in *rhl* system, *rhlI* gene function is encoding the enzyme that is responsible for the production of N-butyryl-homoserine lactone C4-HSL, which binds to RhlR, which acts as a transcriptional regulator to control the

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activity of target promoters.[7] The *rhl* system is responsible for controlling the *las* system at the transcriptional and post-transcriptional levels.[8]

PQS is a quinolone-based Quorum sensing (QS) system that acts through 2-heptyl-3-hydroxy-4-quinolone. PQS connects the LysR-type transcriptional regulator PqsR to stimulate many virulence genes.[9] PQS are believed to create a global regulatory network and are responsible for regulating the expression of up to 12% of the genome of *P. aeruginosa*.[10,11] The objective of this research was to detect the presence quorum sensing genes in *P. aeruginosa,* which is represented by *lasI*, *lasR*, *rhlI*, and *rhlR*, and PQS (*pqsA*, *PgsB, PgsC, PgsD, PgsE,* and *MvfR*) genes, by using PCR technique and interaction between them, after testing their MDR resistant patterns against different antimicrobial drugs.

MATERIALS AND METHODS

Isolation and Identification

267 clinical samples, including burns, wounds, ears swabs, and sputum, were collected from Al-Yarmouk Teaching Hospital, Al Jawadar Hospital, and Medical City, Burn Center in Baghdad, Iraq. Samples were collected between December 2017 to April 2018. 50 *P. aeruginosa* isolates were obtained during this study. VITEK 2 compact system GN cards were carried out to confirm the diagnosis of *Pseudomonas* isolates to species level, according to manufacturer's instructions (Biomerieux, France). Molecular confirmation of the isolates as *P. aeruginosa* was performed by using 16S rDNA, which is a housekeeping gene used for the identification of *P. aeruginosa* at the molecular level. Tables 1 and 2 show 16S rDNA primer annealing temperature, sequences, and the expected size of amplicon for PCR assay.

Antimicrobial Susceptibility Test

The antimicrobial susceptibility test was done by disk diffusion test on Mueller-Hinton agar, using a different antibiotic disk; results, then interpreted as susceptible, intermediate, or resistant to the particular antibiotic, and was obtained as recommended in the Clinical and Laboratory Standard Institute (CLSI), 2016.[12]

The antimicrobials drugs tested were ceftazidime 30 µg, cefepime 30 µg, ciprofloxacin 5 µg, levofloxacin 5 µg, tobramycin 10 µg, amikacin 30 µg, gentamicin 10 µg, piperacillin/carbencillin 100 µg, imipenem 10 µg, azetreonam 30 µg, and colistin sulfate 5 µg (MAST group, UK).

DNA Extraction and Detection of Quorum Sensing Genes by a Conventional Polymerase Chain Reaction

The nucleic acid extraction of the *P. aeruginosa* isolates was performed using a commercial DNA extraction kit (G-spin DNA extraction kit), according to the manufacturer's instruction (Intron Biotechnology). Determination of DNA quality and quantity is done by using nanodrop at 260/280 nm

Table 1: Sequence of primers and molecular size of PCR product

Primer target	*Primers sequence 5′→3′*	*Product bp*	*References*
16S rDNA F	5'- AGAGTTTGATCCTGGCTCAG-3'	1250 base pair	14
16S rDNA R	5'- gGTTACCTTGTTACGACTT-3'		
lasR-F	5'- TGCCGATTTTCTGGGAACC-3'	401 base pair	15
lasR-R	5'- CCGCCGAATATTTCCCATATG -3'		
lasI-F	5'- TCGACGAGATGGAAATCGATG-3'	402 base pair	
lasI-R	5'- gCTCGATGCCGATCTTCAG -3'		
rhlI-F	5'- CGAATTGCTCTCTGAATCGCT -3'	182 base pair	
rhlI-R	5'- gGCTCATGGCGACGATGTA -3'		
rhlR-F	5'- TCGATTACTACGCCTATGGCG -3'	208 base pair	
rhlR-R	5'- TTCCAGAGCATCCGGCTCT -3'		
pgsA-F	5'- CCTGCAATACACCTCGGGTT-3'	898 base pair	This study
pgsA-R	5'- CAGCAGGATCTGGTTTGTCGT-3'		
pgsB-F	5'- TGGCCGACACCCTTTATCACV-3'	407 base pair	
pgsB-R	5'- TCGCGGTTCTCGATCAGATG -3"		
pgsC-F	5'- ACCGTCTGGATGAACTGCTG-3'	289 base pair	
pgsC-R	5'- AGGTGAAGTCGAGCAGGTTG-3'		
pgsD-F	5'- TCCATCCCGTACACCCTGAT-3'	442 base pair	
pgsD-R	5'- AGCAGGTCGAAGTAGTTGCC-3'		
pgsE-F	5'- gGATGCCGAATTGGTTTGGG-3'	317 base pair	
pgsE-R	5'- CTCCATGTCGTCGAACACCA-3'		
mvfR -F	5'- gTTTCGACGAATGCTCGGTTG-3'	302 base pair	
mvfR -R	5'- gACAAGGTGCTCTTCGTGGA-3'		

Table 2: Thermal cycling conditions of *Las1, LasR, rhlI, rhlR, pgsA, PgsB, PgsC, PgsD, PgsE,* and *mvfR* genes used for PCR

Gene	*Initial denaturation*	*No. of cycles*	*Denaturation*	*Primer annealing*	*Primer extension*	*Final extension*
16s RNA	94ºC/3 min	35 amplification cycles	94°C/45 sec	62°C/45 sec	72°C/1 min	72°C/7 min
Lasl						
LasR	95ºC/5 min		95ºC/30 sec	59ºC/1 min	72ºC/80 sec	72ºC/10 min
rhlI						
rhlR	95ºC/5 min		95ºC/30 sec	54ºC/1 min	72ºC/80 sec	72ºC/10 min
pgsA	95ºC/5 min		95ºC/45 sec	60ºC/45 sec	72ºC/45 sec	72ºC/7 min
PgsB	95ºC/3 min		95ºC/45 sec	58ºC/45 sec	72ºC/45 sec	72ºC/7 min
PgsC						
PgsD	95ºC/3 min		95ºC/45 sec	60ºC/45 sec	72ºC/45 sec	72ºC/7 min
PgsE						
mvfR	95ºC/3 min		95ºC/45 sec	57ºC/45 sec	72ºC/45 sec	72ºC/7 min

Table 3: Patterns of resistance of *P. aeruginosa*

Patterns	*Number of antibiotic resistance*	*Isolates number*
A	1–4	34/50 (68%)
B	5–8	11/50 (22%)
C	9–11	5/50 (10%)

and gel electrophoresis.[13] PCR protocol was used to detect the presence of quorum sensing genes *(las1, lasR, rhlI, rhlR, pgsA, PgsB, PgsC, PgsD, PgsE,* and *mvfR*).

Preparation of Primers

las1, lasR, rhlI, rhlR, pgsA, PgsB, PgsC, PgsD, PgsE, and *mvfR* genes were investigated by Integrated DNA Technologies, Inc. (IDT, USA). The lyophilized primers were dissolved in DdH_2O to give a final concentration of 100 pmol/µL, as a stock solution. The stock was kept at -20°C to prepare 10 pmol/µL concentration, as work primer suspended. 10 µL of the stock solution and 90 µL of the DdH_2O water was added to get the final volume (100 µL).

Primers Selection

The primers used in PCR amplification included, 16S rDNA, Las1, LasR, RhlI, RhlR, pgsA, PgsB, PgsC, PgsD, PgsE, and mvfR, as illustrated in Table 1.

RESULTS

Isolation and Identification of *P. aeruginosa*

50 *P. aeruginosa* isolates were obtained in this study. The *P. aeruginosa* isolates were recovered from 28 (56%) male patients and 22 (44%) female patients. These clinical isolates were obtained from burn 24 (48%), wound 12 (24%), sputum 7 (14%), ear 3 (6%), urinary tract infection (UTI) (4%), vaginal 1 (2%), and blood 1 (2%). As shown in Figure 1.

P. aeruginosa isolates were identified biochemically by using VITEK 2 compact system GN cards (BioMerieux, France). According to the 46 biochemical reactions included in cards, all the isolates were identified successfully with an identification probability of 93–99%.

Antibiotic Susceptibility Patterns

The results of antimicrobial susceptibility test rates of the

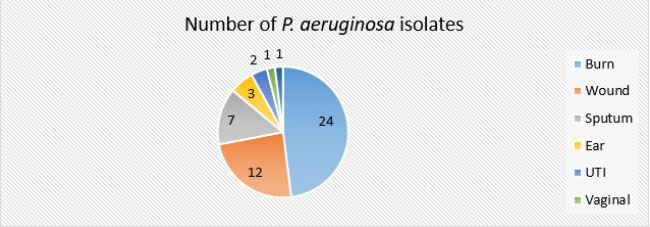

Figure 1: Distribution of *P. aeruginosa* isolates according to clinical sources (N = 50)

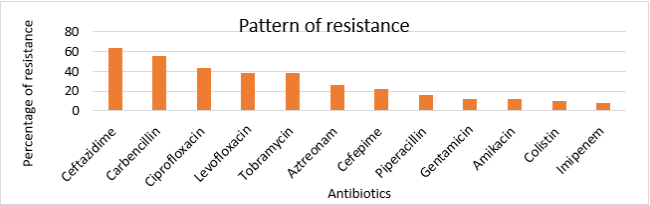

Figure 2: Antibiotic resistance of *P. aeruginosa* isolates

50 *P. aeruginosa* isolates obtained in our study revealed that *P. aeruginosa* was (64%) resistant to ceftazidime, followed by carbenicillin (56%), ciprofloxacin (44%), levofloxacin/tobramycin (38%), aztreonam (26%), and to a lesser extent to cefepime (22%), piperacillin (16%), gentamicin/amikacin (12%), colistin (10%), and imipenem (8%). Resistance rates of *P. aeruginosa* isolated are shown in Figure 2.

P. aeruginosa isolates were differentiated into three different MDR patterns, *viz.*, A, B, and C. Pattern C showed the highest rate of MDR, when bacterial isolates show resistant 9→11 antibiotics, while pattern A isolates showed the lowest MDR, when the isolates were able to resist 1→4 antibiotics only, as shown in Table 3.

Molecular Analysis

The DNA of 50 *P. aeruginosa* isolates were successfully extracted with g-spin DNA extraction kit. Purity and concentration were confirmed with nanodrop, and the intact DNA bands were confirmed through gel electrophoresis.

Identification by using 16S rDNA Gene Analysis

Genotypic identification, depending on 16S rDNA, confirms the diagnosis of the fifty isolates under study, which showed

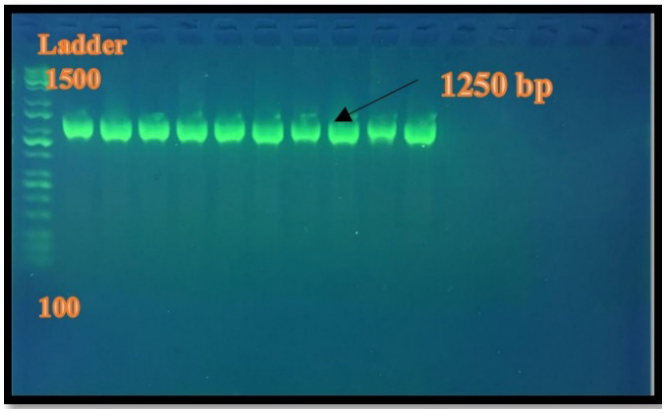

Figure 3: PCR product, band size is 1,250 bp; Product was electrophoresed on 2% agarose at 5 volt/cm^2; 1x TBE buffer for 1:30 hr; N: DNA ladder (100)

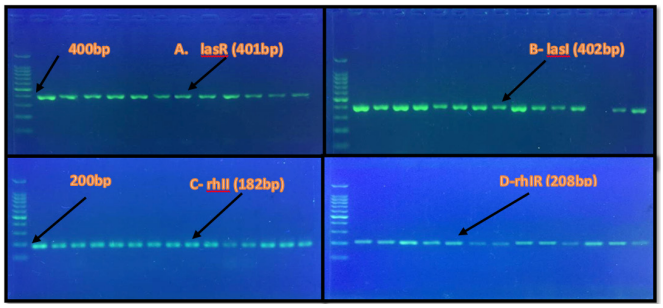

Figure 4: Gel electrophoresis for quorum sensing genes of *P. aeruginosa* isolates; A: for *lasR* gene amplicons (401 bp); B: for *lasI* (402 bp); C: for *rhlI* (182 bp); D: for *rhlR* (208 bp); M: 100 bp ladder; gel electrophoresis was performed using 1% agarose gel, and the run lasted for 50 min/ 100 V

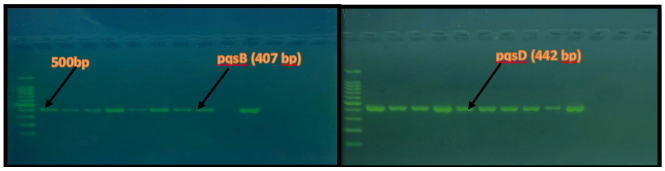

Figure 5: Gel electrophoresis of *pqsA* gene amplicons (898 bp) of *P. aeruginosa* isolates; M: 100 bp ladder; gel electrophoresis was performed using 1% agarose gel, and the run lasted for 50 min

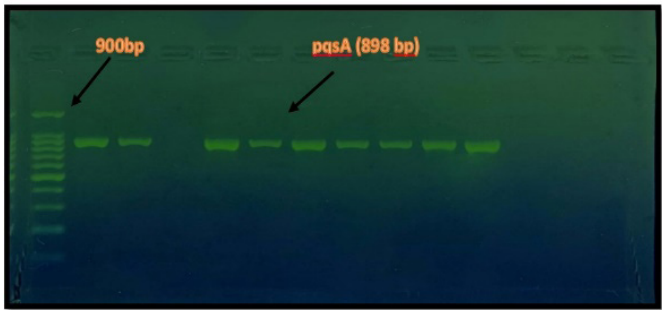

Figure 6: Gel electrophoresis of *pqsB* gene amplicons (407 bp) and *pqsD* gene amplicons (442 bp) of *P. aeruginosa* isolates; M: 100 bp ladder; gel electrophoresis was performed using 1% agarose gel, and the run lasted for 50 min/ 100V

expected amplicons size 1,250 bp (Figure 3).

Detection of QS Genes by Conventional PCR Techniques

The detection of *lasI/lasR* and *rhlI/rhlR* genes showed that all of the 50 isolates under study were positive for one or more QS genes. By PCR technology, result showed that 47/50 (94%) of the isolates were positive for *lasR*, 49/50 (98%) of the isolates were positive for *lasI*, 50/50 (100%) were positive for *rhlI*, 42/50 (84%) of the isolates were positive for *rhlR*, as illustrated in Figure 4.

Twenty *P.aeruginosa* isolates were selected for the detection the present of the third quorum sensing system PQS, which is represented by *pgsA, PgsB ,PgsC, PgsD, PgsE,* and *mvfR* genes, and the result showed that 12/20 (60%) were positive for pqsA, 20/20 (100%) were positive for pqsB, 19/20 (95%) were positive for pqsC, 15/20 (75%) were positive for pqsD, 18/20 (90%) were positive for pqsE, and finally 16/20 (80%) were positive for the receptor *mvfR*, as shown in Figures 5 to 7.

DISCUSSION

As shown in Figure 1, the majority 24/50 (48%) of *P. aeruginosa* isolates were obtained from burns infections. This indicates that *P. aeruginosa* is widely found in hospital environments, such as, distribution systems and air. These observations agree with other authors who mentioned that *P. aeruginosa* is present in about 33% of burn wounds and 59% of extensive burns. Huebinger RM *et al.*[16] and Sallman RS *et al.*[17] noticed that *P. aeruginosa* was obtained from 41.26% burn infection, followed by 28.57% wound swabs. In this study, it was noticed that the lowest percentage of isolation was from vaginal swab 1/50 (2%), followed by blood samples 1/50 (2%). Even when the isolation percentage is low, it can still cause a high mortality rate in these sites comparing with other gram-negative bacteria.[18]

Molecular identification based on 16S rDNA amplification protocol for *P. aeruginosa*, including PCR assays and DNA amplification by using standard forward and reverse 16S universal primers. The taxonomic gold standard for the determination of the phylogenies of bacterial species and the identification of bacteria, is by using 16S rDNA, which is a housekeeping gene.[19] Our observation showed the utility of 16S rDNA PCR amplification. This reveals the high specificity of the primers used in the study and the accuracy of the PCR machine.

The susceptibility pattern test of *P. aeruginosa* bacteria under study suggests the idea that they might harbor different antibiotics resistant mechanisms against ceftazidime, followed by carbencillin antibiotics, which show a high resistant rate 64 and 56%, respectively, Figure 2, Results are in agreement with Othman N *et al.*,[20] who reported the resistant rate of ceftazidime was 69.2%, and Ronat JB *et al.*,[21] who found out that the resistant rate for carbencillin was 54%. Only five *P. aeruginosa* isolates in this study belong to MDR pattern C, as shown in Table 3. Different genetic events, like the acquisition of different mutations or horizontal transfer of genes responsible for antibiotic resistance, are responsible for the development of MDR by *P. aeruginosa* isolates.[22] It is worth mentioning that in this study, 90% *P. aeruginosa*

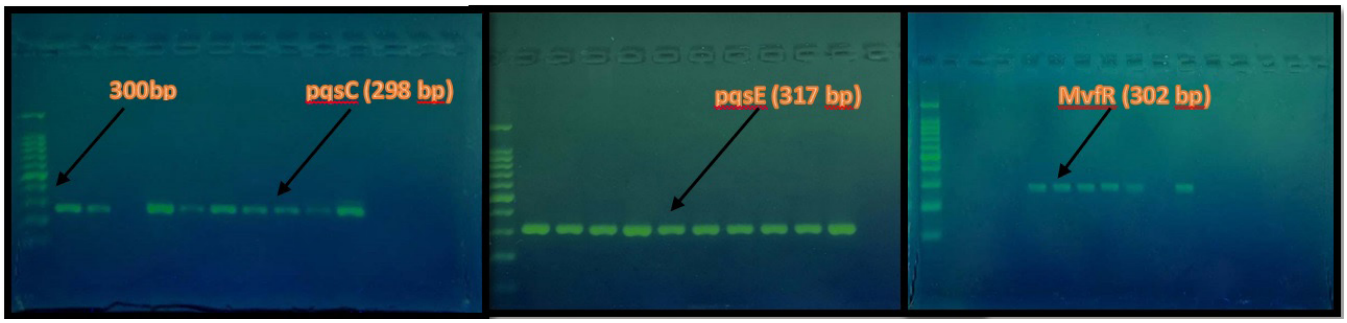

Figure 7: Gel electrophoresis of *pqsC* gene amplicons (298 bp), *pqsE* gene amplicons (317 bp), and *mvfr* gene amplicons (302 bp) of *P. aeruginosa* isolates; M: 100 bp ladder; gel electrophoresis was performed using 1% agarose gel, and the run lasted for 50 min/ 100V

Table 4: Distribution of quorum sensing genes among *P. aeruginosa* isolates

Gene patterns	*Quorum sensing gene detected by PCR*	*Isolates number*
A	*lasI, lasR/ rhlI, rhlR/ MvfR, pqsA, pqsB, pqsC, pqsD, pqsE*	2, 3, 4, 5, 6, 9, 12, 16
B	*rhlI/MvfR, pqsA, pqsB, pqsC, pqsD, pqsE*	13, 14, 18
C	*lasI, lasR/ rhlI, rhlR/ pqsB, pqsE*	7, 10, 11
D	*lasI, lasR/ rhlI, rhlR/ pqsB, pqsC*	17, 19
E	*lasI, lasR/ rhlI, rhlR/ MvfR, pqsB, pqsC, pqsD, pqsE*	1, 8, 15, 20

isolates were susceptible to the imipenem, as shown in Figure 2. Al-Charrakh AH *et al.*[23] observed similar data, while Hussein ZK *et al.*[24] and Amini A *et al.*[25] reported that *P. aeruginosa* had an intermediate resistance (35 and 39.3%, respectively) to imipenem.

PCR analysis of *P. aeruginosa* under study for the presence of QS genes, indicates that they can have different patterns. According to quorum sensing genes production, gene pattern A includes the *P. aeruginosa* isolates 2, 3, 4, 5, 6, 9, 12, and 16, were all positive for *las* and *rhl* systems, as shown in Table 4. Previous study showed that *las* and *rhl* system is the first QS system discovered in *P. aeruginosa*, which is controlled via the AHL,[19] by comparing these result with the antibiotic susceptibility patterns, Table 3. It is worth mentioning that the *P. aeruginosa* isolates number 2, 3, 4, 5, 6, 9, 12, and 16, which were positive for *las* and *rhl* system, showed high resistant to 5 to 10 of the antimicrobial agent under study. This is in agreement with a result of previous researchers who observed a relationship between *las* and *rhl* system, and regulation of antibiotics resistance via efflux pump genes.[26] Another study showed that clinical *P. aeruginosa* isolates were deficient in QS genes, and were generally less susceptible to antibiotics.[27] As shown in Table 4, gene pattern A, the PQS biosynthetic genes *pqsABCDE* are all present, specially *MvfR* gene, which is considered a master regulator of PQS biosynthesis genes.[28] There is a relationship between *lasR*/3-oxo-C12- and PQS biosynthesis; *lasR* considered as positive regulator of PQS biosynthesis,[29] and that is the main reason why all the *pqsABCDE* were turned on, *MvfR* will positively regulate the transcription of the *pqsABCDE* and *phnAB* operons directly.[29]

Our result also showed that gene pattern B, which include the isolate number 13, 14, and 18, Table 4, has a defect for the production of *lasR, rhlR* genes, which are considered as transcription regulators of quorum system that when companied with their specific autoinducers, activate transcription of different virulence factors.[30] This defect can be caused by mutation or other reason that cause the genes to be silent, while the same isolates have the PQS system, which includes *pqsABCDE MvfR* genes, Table 4. It can be concluded that *P. aeruginosa* isolates find their way for the second quorum system, *viz.*, PQS, to express their virulence factor. As previously concluded by Diggle SP *et al.*,[31] known that the regulation of PQS biosynthesis by LasR (3-oxo-C12-AHL) occurs through transcriptional regulation of MvFR that is responsible for converting HHQ to PQS,[32] but under certain growth situation, there can be a considerable LasR independent biosynthesis of PQS genes.[31] Another evidence that isolates 13, 14, and 18 have the PQS fully functional is their high resistance to fluoroquinolones family (ciprofloxacin and levofloxacin), as shown in Figure 2; this finding is in agreement with Heeb S *et al.*[33] In spite of the deficient in lasR, rhlR isolates 13, 14, and 18, they still have the ability to resist to some antibiotics in addition to their biofilm formation and pigment production abilities. All these virulence factors under the control of *las*, *rhl* systems, our explanation that a *lasR*, *rhlR* deficient strain may lead to cause infection by the presence of multiple *P. aeruginosa* bacteria in infection site. The patient could be infected by both QS deficient and proficient strains of *P. aeruginosa*. QS deficient strains could profit from the extracellular enzymes produced by QS proficient, and this can lead to a QS deficient strain to take part in infection.[34] In the same manner, Ali MR *et al.*[35] indicates the low or moderate resistance to antibiotics focused in isolates with high genetic content.

Our result also revealed that isolates number 7, 10, and 11 within gene pattern C, and isolates number 17 and 19 within gene pattern D, were positive for production *las* and *rhl* systems, Table 4, but has a defect for the production of *MvfR* gene. Even some of the PQS genes were turned on,

knowing that these isolates still require *MvfR* gene as a transcriptional regulator for PQS biosynthesis.[32,36] While pattern E isolates number 1, 8, 15, and 20 have only defective in *pqsA* gene production, *pqsA* is an anthranilate coenzyme A, has an important role in PQS biosynthesis.[37]

CONCLUSION

It was concluded that the quorum-sensing systems in *P. aeruginosa* play a main role in the pathogenesis of infections. It is also concluded that *P. aeruginosa* isolates, which have all the QS genes, tend to be highly susceptible to antimicrobials agents. Our study revealed that the regulation PQS system was mediated by the *las* and *rhl* system. These findings suggest PQS can act as an intercellular signal in *P. aeruginosa* that is not restricted only to AHL. This can be an important tool for further future studying of quinolone signaling in more specific in *P. aeruginosa* bacteria.

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