Egyptian Journal of Medical Microbiology Volume 26 / No.3 / July 2017 11-18

ORIGINAL ARTICLE

The Role of Quorum Sensing Genes (*las* and *rhl*) in Biofilm Forming *Pseudomonas aeruginosa* Isolates

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Key words:
Pseudomonas aeruginosa; biofilm; autoinducers; quorum sensing; las and rhl system

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ABSTRACT

Background: *Quorum sensing (QS) is a bacterial cell-cell communication process that involves the production, detection, and response to extracellular signaling molecules called autoinducers (AIs). Processes controlled by QS include bioluminescence, sporulation, competence, antibiotic production, biofilm formation, and virulence factor secretion. Pseudomonas aeruginosa (P. aeruginosa) produces multiple virulence factors and causes different types of infections.* ***Objectives:*** *to determine if deficiency within the QS system decreases the ability of P. aeruginosa to establish biofilm formation and infections in humans or not.* ***Methodology:*** *This was achieved through determination of the levels of P. aeruginosa autoinducers as well as the presence of QS-controlled genes (lasI, lasR, rhlI and rhlR) within the biofilm-forming clinical samples.* ***Results:*** *We collected 186 P. aeruginosa isolates, 89.3% were significantly biofilm producer strains. About 66% were strong, 28% were moderate and 15% were weak biofilm producer clinical isolates. Autoinducers evaluation revealed that, 8-clinical isolates (CIs) for $3OC_{12}$-HSL and/or C_4-HSL concentration were deficient. The amount of $3OC_{12}$-HSL produced by 8-CIs was significantly lower than that produced by PAO1. While 6-CIs produced no C_4-HSL and 2-CIs produced detectable levels of C_4-HSL. Polymerase chain reaction (PCR) analysis of autoinducers deficient CIs revealed that, two isolates contained all four QS genes (lasI, lasR, rhlI and rhlR) and were moderate-biofilm producers while three isolates lacked all QS genes and were non-biofilm producer strains. Analysis of remaining autoinducers deficient CIs revealed that, they were weak-biofilm producer strains and they had intact las system in two strains and lacked lasR in third one while they were completely lacked the rhl system. Patients had autoinducers deficient CIs were isolated from cardiac catheterization, tracheostomy tubes, sputum, and wound infections.* ***Conclusion:*** *Naturally occurring QS-deficient strains of P. aeruginosa were capable of causing different infections despite the loss of optimum gene. QS-deficient genes affected the severity of biofilm formation. So the development of efficient cell-to-cell signaling blockers may reduce P. aeruginosa biofilm formation on medical devices.*

INTRODUCTION

P. aeruginosa is a Gram-negative bacterium capable of surviving in a wide range of environments. It is an opportunistic pathogen and it is commonly associated with nosocomial infections and infections of severely burned individuals, and are a leading cause of death in severe respiratory infections[1]. In particular, P. aeruginosa is the leading cause of chronic pulmonary infections and mortality in cystic fibrosis (CF) patients[2]. Infections with *P. aeruginosa* are difficult to eradicate, due to their high levels of antibiotic resistance and growth in biofilms[3].

Bacterial communication occurs through a well-developed system termed the quorum sensing (QS) system. QS system is the regulation of gene expression in response to fluctuations in cell-population density. Quorum sensing bacteria produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density. Cell-cell communication *via* autoinducers occurs both within and between bacterial species. The detection of a minimal threshold stimulatory concentration of an autoinducer leads to an alteration in gene expression. Bacteria use quorum sensing communication circuits to regulate a diverse array of physiological activities such as virulence, competence, conjugation, antibiotic production, sporulation, motility, biofilm formation and symbiosis[4, 5, 6].

Quorum sensing was originally described in the marine luminescent bacterium *Vibrio fischeri* that control the light production[7]. For years, QS researches were restricted to a few marine organisms but it is now widely recognized that many bacterial species utilize QS as part of their regulatory machinery[8]. This has led to a burst in QS research and in its role in the virulence of multiple human pathogens, which has been studied in molecular detail.

P. aeruginosa possesses two well-defined, interrelated QS systems, *las* and *rhl*. Each QS system consists of two components, the autoinducer synthases (*LasI* and *RhlI*) and their cognate transcriptional regulators (*LasR* and *RhlR*, respectively)[9, 10]. *LasI* synthesizes the autoinducer N-(3-oxododecanoyl) homoserine lactone ($3OC_{12}$-HSL), while *rhlI* synthesizes the autoinducer N-butyryl homoserine lactone (C_4-HSL). At high cell density, $3OC_{12}$-HSL and C_4-HSL reach critical levels and activate their regulators, which in turn enhance the transcription of different virulence genes[11].

Clinical studies suggested that QS systems are fully functional within infected lungs of cystic fibrosis patients, who were chronically infected with *P. aeruginosa*[12]. Analysis of patients sputa revealed the presence of both *lasI* and *lasR* transcripts[13]. Other studies upon different animal models of *P. aeruginosa* infections including the thermally injured mouse model and the mouse models of acute and chronic lung infections revealed the importance of QS in the virulence of *P. aeruginosa* [14,15,16,17]. These studies compared the virulence of *P. aeruginosa* mutants that carried deletions within QS genes with that of their parent strain. They showed that the mortality rate among thermally injured mice infected with QS mutants was significantly lower than that in mice infected with the parent strain. In addition, the mutants were significantly defective in their ability to spread either locally within the thermally injured skin or systemically within the bodies of the thermally injured/infected mice.

Many bacteria are capable of forming biofilms, and *P. aeruginosa* is one of the most commonly studied. Recent work has begun to uncover some of the genetic and molecular mechanisms underlying biofilm production by this organism. *P. aeruginosa* QS signal molecules (autoinducers) play an important role in the biofilm production and differentiation process[18].

Since the QS systems control the production of different virulence factors and has a role in bacterial pathogenicity, it is possible that the loss of one or both systems severely compromises the ability of *P. aeruginosa* to cause biofilm and persistent infections in humans. In this study, we tried to determine if QS-deficient strains of *P. aeruginosa* capable of causing biofilm, the rate at which it occurs and severity of biofilm, and if its occurrence is associated with specific types of infections. Moreover, discuss the possibility of developing new strategies to control bacterial virulence using QS inhibitors.

METHODOLOGY

Clinical isolates:

The study was conducted during the period from December 2013 to January 2016. Permission of the patients and approval from the local ethic committee were obtained for the use of the specimens. One hundred eighty six *P. aeruginosa* isolates were obtained from specimens collected from patients in different hospitals in Riyadh, Saudi Arabia. The isolates were obtained from specimens from clinically diagnosed infected endotracheal tubes, cardiac catheterization, tracheostomy tubes, wounds, burns, sputa from patients with lower respiratory tract infection, infected urine, ear discharge and others. Only one isolate for patient was considered in order to avoid duplicates. The isolates were identified according to standard methods[19]. The isolates were streaked on Luria-Bertani (LB) agar and stored in 20% glycerol/LB broth at -80 °C. These stock cultures were used to inoculate media for different assays to avoid repeated subculturing of the isolates.

Analysis of static biofilm formation:

Sterile polyvinyl chloride (PVC) pieces of approximately $1cm^2$ were cut from original urinary catheter and biofilm was allowed to develop under static conditions for 5 days. Briefly, bacteria were grown overnight on pseudomonas agar (Oxoid) plates and subcultured onto trypticase soy agar (TSA) (Oxoid) plus 5% glucose. Bacteria were then resuspended in trypticase soy broth (TSB) plus 5% glucose. The optical density at 650nm (OD 650) of the bacterial suspensions was determined and PVC pieces were immersed in the bacterial culture medium and incubated for 5 days without agitation at 37 °C. After the incubation period, the PVC pieces were individually washed twice in 0.9% NaCl and subjected to biofilm measurements by crystal violet staining solution (0.1% in distilled water) for 15min, and washed three times in distilled water. The stain was then dissolved in ethanol and absorbance was measured at 570nm[20]. Interpretation of biofilm production was according to the criteria of Stepanovic et al.[21]. PAO1 was used as a positive control for biofilm-forming strain[22].

Autoinducer measurements:

Cross-feeding bioassay for $3OC_{12}$-HSL and C_4-HSL was done as described previously with some modifications[22, 23]. For extraction of autoinducers from biofilms, polyvinyl chloride (PVC) fragments on which biofilms had been formed were rinsed three times and immersed into 1ml of 0.9% NaCl. One volume of acidified ethyl acetate (0.01% acetic acid) was added and the mixture incubated in closed tubes at 4 °C overnight. A second extraction was carried out for 1h, and the two extracts were pooled. The extracted autoinducers were quantified using specific bioassays using strain *Agrobacterium tumefaciens* NTL4 (BAA-2240™), which contains two plasmids; one carries the traR gene and the other carries the traI–lacZ fusion for $3OC_{12}$-HSL and *P. aeruginosa* PAO-JP2 (carrying plasmid pECP61.5) strains for C_4-HSL. In the presence of autoinducers in the tested supernatant samples, the levels of β-galactosidase activity were enhanced. Briefly, NTL4 and PAO-JP2 were grown overnight at

30 °C in LB broth. Samples of the overnight cultures were diluted to an OD_{600} of 0.1 and 0.05 for NTL4 and PAO-JP2 strains, respectively. A 400 µl aliquot of each tested supernatant sample was added to 5ml of the diluted NTL4 and PAO-JP2 cultures and the cells were grown at 30 °C for 24 h. The cells were then pelleted, suspended in 200µl distilled water and sonicated (Branson Ultrasonic Cleaner; Branson Cleaning Equipment Company). β-Galactosidase (β-gal) activity was determined as Miller units as previously described by Miller[24].

PCR analysis of the QS genes:

Chromosomal DNA was extracted from PAO1 wild-type and from the autoinducers deficiency clinical isolates and utilized as templates in PCR experiments[25]. DNA was extracted using illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare, UK). A set of oligonucleotide primers was designed by Eurofins MWG Operon (Germany) corresponding to different regions among intact gene amplicons of *lasI, lasR, rhlI* and *rhlR* genes and was synthesized **(Table 1)**. PCR was carried out in a total volume of 45µl with 30ng chromosomal DNA as a template. PCR conditions included: heating at 94°C for 15 min; followed by 34 cycles of 94 °C for 30s, 59°C for 30s and 72 °C for 2min; and a terminal cycle of 72 °C for 10min using Mycycler TM Thermal cycler (BioRad, USA). Synthesized DNA fragments were detected on 1.5% agarose gels by ethidium bromide staining.

Table 1: The primers utilized in PCR experiments for intact amplicons of the QS genes

Gene	*Sequence*	*Length of Primer (bp)*	*Size of Amplified Product (bp)*
QS gene amplicons:			
lasI-f	5' ATGATCGTACAAATTGGTCGGC 3'	**22**	**605**
lasI-r	5' GTCATGAAACCGCCAGTCG 3'	**19**	
lasR-f	5' ATGGCCTTGGTTGACGGTT 3'	**19**	**725**
lasR-r	5' GCAAGATCAGAGAGTAATAAGACCCA 3'	**26**	
rhlI-f	5' CTTGGTCATGATCGAATTGCTC 3'	**22**	**625**
rhlI-r	5' ACGGCTGACGACCTCACAC 3'	**19**	
rhlR-f	5' CAATGAGGAATGACGGAGGC 3'	**20**	**730**
rhlR-r	5' GCTTCAGATGAGGCCCAGC 3'	**19**	

f, forward; r, reverse.

RESULTS

One hundred eighty-six isolates were identified as *P. aeruginosa*. The isolates were obtained from specimens of infected endotracheal tubes, cardiac catheterization, tracheostomy tubes, wounds, burns, sputa from patients with lower respiratory tract infection, infected urine, ear discharge and others. All isolates were screened for ability of biofilm formation and for the concentration of autoinducers ($3OC_{12}$-HSL and C_4-HSL) in culture supernatants. The production of autoinducers is stringently controlled by the QS system. Therefore, the strains that had autoinducers deficient and suspicious to carry deletion in the *las* and/or *rhl* gene were subjected for further molecular study.

***P. aeruginosa* isolates and site of infections:**

Table 2 summaries the correlation between the number of the isolates and site of *P. aeruginosa* infections. The highest number of *P. aeruginosa* was isolated from infected surgical or non-surgical wounds (17.7%) followed by isolates from burn (15.6%), tracheostomy tubes (14%), endotracheal tubes (12.4%) and sputa (11.3%). There were no significant differences between the number of the isolates and site of *P. aeruginosa* infections (P > 0.05).

Table 2: Correlation between the number of the isolates and site of *P. aeruginosa* infections

Site of specimens	*Number of isolates (%)*	*P value*
Endotracheal tubes	23 (12.4)	
Cardiac catheterization	12 (6.5)	
Tracheostomy tubes	26 (14)	P >
Wound	33 (17.7)	0.05
Burns	29 (15.6)	
Sputa	21 (11.3)	
Urine	17 (9.1)	
Ear discharge	16 (8.6)	
Others	9 (4.8)	
Total	186 (100%)	

Static biofilm formation profile:

Regarding Stepanovic et al. criteria[21], quantity of biofilm production profile was done **(Table 3)**. The strongly-biofilm producer isolates were represented by 66.1% of the isolates while non-biofilm producers were represented by only 10.7% of the studied isolates. Weakly and moderately-biofilm producers were 8.1% and 15.1%, respectively. There was a significant different between degrees of biofilm producer strains and between the biofilm producer group and non-biofilm producer group (P<0.001 and P<0.05, respectively).

Table 3: Classification of biofilm production of bacteria based on optical density measured at OD_{570} by ELISA reader

	Biofilm profile			
	Non- biofilm producer	Biofilm producer		
		Weak	Moderate	Strong
Average OD parameters	≤ ODc	ODc < ~ ≤ 2x ODc	2× ODc < ~ ≤ 4x ODc	> 4x ODc
Average OD results	OD ≤ 0.163	0.163 < OD ≤ 0.326	0.326 < OD ≤ 0.652	> 0.652
Number of isolates (%)	20 (10.7)	15 (8.1)	28 (15.1)	123 (66.1)
Total profile (%)	20 (10.7)	166 (89.3)		
P value	-	P < 0.001		
		P < 0.05		

Note: optical density cut-off value (ODc) = average OD of negative control + 3SD of negative control

Production of autoinducers by clinical isolates:

The study revealed that, 8-deficient clinical isolates (CIs) for $3OC_{12}$-HSL and/or C_4-HSL concentration. The positive control strain, PAO1, usually produces a considerable amount of the autoinducers **(Figure 1a and 1b)**. As shown in figure 1a, the amount of $3OC_{12}$-HSL produced by the 8-CIs was significantly ($P < 0.05$) lower than that produced by PAO1. Figure 1b showed that, CI-1, CI-2, CI-3, CI-5, CI-6 and CI-8 produced no C_4-HSL. However, CI-4 and CI-7 produced detectable levels of C_4-HSL. These results suggested that CI-1, CI-2, CI-3, CI-5, CI-6 and CI-8 were defective in the production of both autoinducers ($3OC_{12}$-HSL and C_4-HSL) while CI-4 and CI-7 were completely defective in the production of $3OC_{12}$-HSL but produce considerable amount of C_4-HSL.

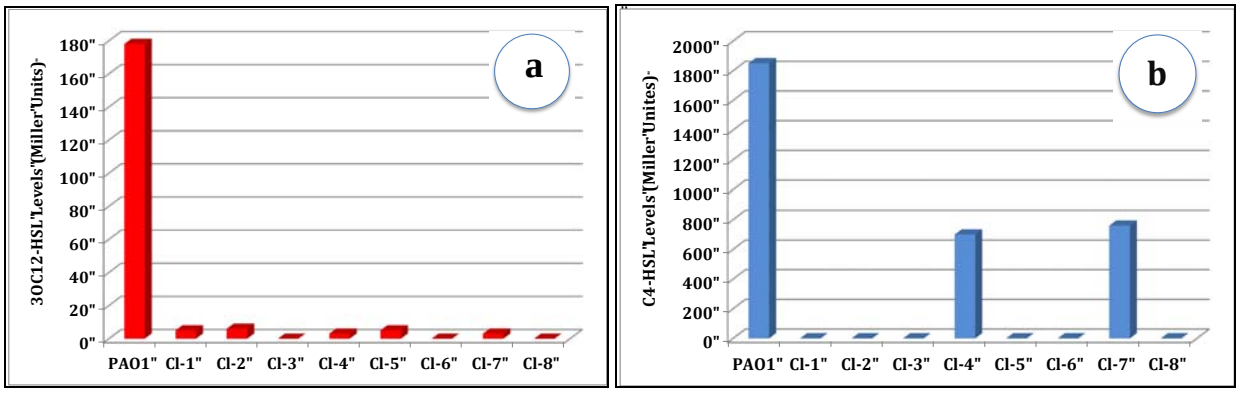

Fig. 1: Levels of $3OC_{12}$-HSL (a) and C_4-HSL (b) present in the supernatants of 8 clinical isolates and controls

Autoinducers and biofilm profile of the QS-deficient *P. aeruginosa* clinical isolates:

Table 4 summaries the correlation between autoinducers levels and biofilm profile of the different phenotypes of *P. aeruginosa* clinical isolates. Among the 8-deficient CIs for $3OC_{12}$-HSL and/or C_4-HSL concentration there were; 3 non-biofilm producers strains with no detectable any autoinducers (CI-3, CI-6 and CI-8), 3 weak-biofilm producers strains with little amount of $3OC_{12}$-HSL and no detectable C_4-HSL (CI-1, CI-2 and CI-5) and 2 moderate-biofilm producers strains with little amount of $3OC_{12}$-HSL and considerable amount of C_4-HSL (CI-4 and CI-7). No strong-biofilm producers strains among the 8-deficient CIs of autoinducers. No significant correlation between the site of *P. aeruginosa* infection and severity of biofilm formation or levels of autoinducers ($P > 0.05$).

Table 4: Correlation between site of *P. aeruginosa* infections, autoinducers and biofilm profile of the different phenotypes of *P. aeruginosa* clinical isolates

Strain	*P. aeruginosa* infection site	Biofilm Formation		3OC12 –HSL (Miller units)*	C4 –HSL (Miller units)*	P value
		% of PAO1	Severity of biofilm			
CI-1	Tracheostomy tubes	7	Weak	4	0	> 0.05
CI-2	Sputum	10	Weak	6	0	
CI-3	Wound	0	No biofilm	0	0	
CI-4	Cardiac catheterization	23	Moderate	3	39	
CI-5	Sputum	9	Weak	6	0	
CI-6	Sputum	0	No biofilm	0	0	
CI-7	Wound	29	Moderate	4	42	
CI-8	Tracheostomy tubes	0	No biofilm	0	0	

* Results are given as percentages of the activity of PAO1, the 100 % control

Analysis of the QS genes:

The 8 CIs with reduced or absent autoinducer activity were subjected to PCR analysis of QS genes. The failure or reduction of autoinducers levels may be due to the loss of any one of the genes that code for different components of the QS systems. We examined this possibility by attempting to synthesize DNA fragments carrying intact *lasI, rhlI, lasR* and *rhlR*. PAO1, which carries intact QS genes, was used as a positive control **(figure 2)**. As shown in figure 2a, a 605 bp fragment that carries intact *lasI* fragment was synthesized from the chromosome of PAO1 and CI-1, CI-2, CI-4, CI-5 and CI-7. Regarding *lasR* gene (figure 2b), all CIs revealed the same results as *lasI* gene except CI-2 that showed no amplified gene band. With respect to *rhlI* and *rhlR* (figures 2c and 2d, respectively), fragments that carry intact *rhlI* and *rhlR* were synthesized from the chromosome of PAO1 strain and CI-4 and CI-7 only at 625 bp and 730 bp, respectively.

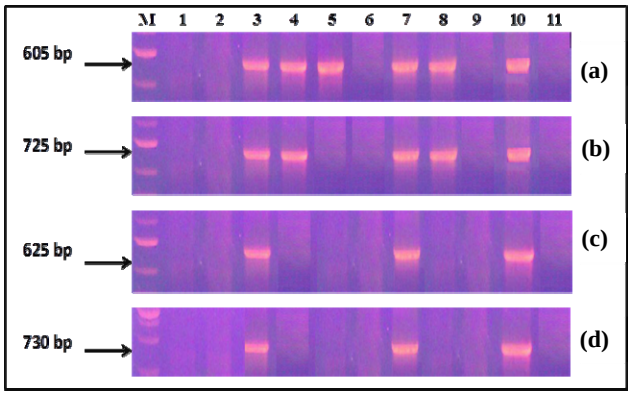

Fig. 2: Ethidium bromide-stained 1.5% agarose gels showing the amplified QS genes of 8-deficient autoinducers clinical isolates and PAO1 strain by PCR. (a) lasI; (b) lasR; (c) rhlI and (d) rhlR genes. Lanes: (M) 1000 bp molecular size standard; (1) empty well; (2) no template control sample; (3) PAO1, positive control strain; (4 –11) CI-1 through CI-8. Arrows indicate amplicons for each gene. Sizes of relevant molecular size standards and the amplicons are given in bp.

DISCUSSION

Regulation of biofilm formation requires a better understanding of the molecular mechanisms underlying the complex and dynamic processes of biofilm development. A number of studies had linked QS and biofilm formation/development in *P. aeruginosa*[26, 27, 28, 29], although some studies indicated that QS had little or no role, with QS mutants being proficient in biofilm formation[30, 31, 32]. In our study, we tried to support one direction of these discrepancies. This was achieved through determination of the levels of *P. aeruginosa* autoinducers as well as the presence of QS-controlled genes (*lasI, lasR, rhlI* and *rhlR*) among the biofilm-forming clinical samples. Our results revealed that, 89.3% of our isolates were biofilm producer strains (about 66% were strong, 28% were moderate and 15% were weak biofilm producer CIs) and 10.7% were non-biofilm producer strains. Eight-CIs were deficient for $3OC_{12}$-HSL and/or C_4-HSL. PCR analysis of autoinducers deficient CIs revealed that, two isolates contained all four QS genes (*lasI, lasR, rhlI* and *rhlR*) and were moderate-biofilm producers while three isolates lacked all QS genes and were non-biofilm producer strains. This is in agreement with the results of Li et al.[33], who found that QS, especially the *Las* system, is critical for biofilm formation and thus for the bacterial parasitism of human hosts and with the data of O'Toole and Kolter[34], who found that *LasI* mutation in *P. aeruginosa* resulted in a formation of defective flat, uniform undifferentiated biofilms lacking mature three-dimensional structure.

In our study, three autoinducers deficient-CIs out of eight-CIs revealed that, they were weak-biofilm producer strains and they had intact *las* system except *lasR* gene was lacked in one strain and they were completely lacked the *rhl* system. As in the study of Christensen et al.[35], he showed that in a *P. aeruginosa rhlI* mutant, the biofilm volume was reduced by 70%. These results suggested that, the development and the severity of biofilm formation were affected by QS system. The *las* system exerts a positive control over the

rhl system, inducing both *rhlI* and *rhlR* transcription, and therefore sits at the upper level of the regulation circuit[36]. The presence of *rhl* system affected the severity of biofilm but not affected the initiation of its formation. The absence of *lasR* in one strain may be due to point mutation in the gene that interfered with the hybridization of the primers and prevented the synthesis of the PCR products because we used primers for intact amplicons of the QS genes. This can be confirmed by using specific primers for internal QS genes. Schaber et al.[37], study, cited the same situation. Also, the study of Davies et al.[38], revealed that, the *las* QS system, but not the *rhl* QS system, was important for *P. aeruginosa* biofilm development.

Several studies have published findings supporting a role for QS, including the *rhl* QS system, in *P. aeruginosa* biofilm formation and some studies have not support this link. The most plausible explanation for these discrepancies is that different experimental parameters as differences in biofilm model and/or culture conditions. A recent study confirmed that, the QS dependence of biofilm formation is nutritionally conditional. Biofilms formed by QS mutants were quite different from those of the PAO1 parent when grown on succinate, but not glucose or glutamate. Under succinate conditions, the mutant biofilms contained microcolonies; whereas the wild-type biofilm was flat and uniform[39]. It should be mentioned here that other studies have shown that growth conditions influence both timing and overall expression of the *las* and *rhl* QS systems[40]. These findings should be considered during the design and interpretation of *P. aeruginosa* biofilm studies since factors that influence biofilm formation under one set of conditions may not do so under all circumstances.

In our study, patients' samples had autoinducers deficient-CIs were isolated from cardiac catheterization, tracheostomy tubes, sputum, and wound infections. These results suggested that naturally occurring QS-deficient strains of *P. aeruginosa* were capable of causing different infections despite the loss of optimum gene. This can be explained by the compensatory effects of other virulence factors for the loss of any single virulence factor (QS system). Many studies supported our results[41, 42, 43, 44, 45].

CONCLUSION

In conclusion, the QS system plays an important role in P. aeruginosa biofilm formation. These findings make QS genes attractive targets for antimicrobial therapy. So the development of efficient cell-to-cell signaling blockers may reduce *P. aeruginosa* biofilm formation on medical devices.

The QS systems are not absolutely essential for *P. aeruginosa* to establish infection, and that other QS independent virulence factors can substitute for the loss of QS-controlled virulence factors.

Acknowledgement

Thanks to pharmacist Mona Al-Semary and Dr. Maha Abdullah, Microbiology department, King Saud University, Saudi Arabia, for providing us with the primers and control strains

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