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ORIGINAL ARTICLE

Quorum sensing-dependent virulence factors and biofilm formation of *Pseudomonas aeruginosa* isolates from retrieved orthopedic implants

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ABSTRACT

Background: *Quorum sensing (QS) plays a crucial role in medical device-related infections. However, epidemiological analysis of QS- dependent virulence factors among Pseudomonas aeruginosa (P. aeruginosa) isolates from orthopedic implants-related infections has rarely been reported.* ***Objective:*** *to assess some QS-dependent virulence factors (as pyocyanin, elastase, protease, rhamnolipid, and biofilm production) in P. aeruginosa isolates from retrieved different orthopedic implants.* ***Methodology:*** *Twenty-four P. aeruginosa isolates obtained from 300 retrieved orthopedic implants with proven diagnosis of osteoarticular infection directly related to orthopedic implants were investigated for production of QS-dependent virulence factors. The pyocyanin production was assayed using King A media, rhamnolipid production was checked utilizing cetyltrimethylammonium bromide (CTAB) agar test. Elastin lytic activity was assayed using elastin Congo red, total protease was estimated using the modified skimmed milk assay technique, and the biofilm forming ability was assayed by microtiter plate assay. N-acyl homoserine lactones (3-oxo-C_{12}-HSL, C_4-HSL) were detected by Cross-feeding bioassay. Polymerase chain reaction (PCR) was used to assess the presence of the QS genes lasI, lasR, rhlI, and rhlR in P. aeruginosa isolates.* ***Results:*** *Seventeen isolates (70.8%) displayed the QS-dependent phenotypes and seven isolates were found to be lacking all tested virulence factors. The lack of production of pyocyanin, Rhamnolipid, las B elastase, total protease, and biofilm formation by the seven isolates proposed that they might have a defective QS system. Among the Seven isolates exhibiting impaired QS-dependent phenotypes, AHLs autoinducers were present in three isolates, though the residual four isolates were lacking both autoinducers and therefore are considered QS-deficient. PCR analysis declared that two isolates contained lasI, lasR, rhlI and rhlR genes while one isolate lacked lasR gene, and one isolate was lacking the four tested genes.* ***Conclusion:*** *Although QS seems to be a crucial regulator of virulence factors and biofilm formation in P. aeruginosa, it is equipped to produce implant infection in humans despite an impaired QS system. These results do not challenge the concept that QS plays a foremost role in P. aeruginosa pathogenicity, but highlight that along with known virulence factors, perhaps there are other virulence factors, that may not be strictly under QS control.*

INTRODUCTION

Implant- associated infections are inherent in substantial morbidity and enormously high cost. *Pseudomonas aeruginosa (P. aeruginosa)* is the commonest Gram-negative bacteria associated with infections of foreign body devices and indwelling catheters [1].

Irrespective of the implant type, *P. aeruginosa* had been previously reported as the cause of infections in 4 to 6% of infected orthopedic implants [2].

P. aeruginosa is standout amongst the most widely recognized opportunistic human pathogens because it employs numerous virulence factors and pathogenicity tools during infection [3].

P. aeruginosa has a remarkable buildup of both extra-cellular and cell-associated virulence factors. Factors associated with extra-cellular virulence and intracellular communication are not constitutively expressed but somewhat cell-density dependent, and are altogether regulated by cell-to-cell signaling i.e. well-defined quorum-sensing (QS) systems that controls expression of many virulence factors, besides biofilm formation[4]. Briefly, These cell-to cell signaling systems are all composed of a small diffusible molecule, called an autoinducer (N-acyl-l-homoserine lactones (AHLs)), which is synthesized by an autoinducer synthase, and a transcriptional activator protein[5]. The autoinducer, therefore, allows the bacteria to communicate with each other, to sense their own density, and together with a

transcriptional activator to express specific genes as a population rather than individually thus it couples gene expression with cell-population density.

Two entangled *QS* systems have been displayed to be associated with virulence, biofilm formation, and extra mechanisms involved in *P. aeruginosa* pathogenicity. *Las* system comprises a *lasI* -encoded acyl-HSL synthase and the *lasR*-encoded transcriptional activator [6]. *Rhl* system comprises *rhlI*-encoded acyl-HSL synthase and *rhlR*-encoded transcriptional activator [7]. In the relevant *Q*S systems each yield and respond to a precise acyl-HSL; *LasI* guides the synthesis of 3-oxo-dodecanoyl-HSL ($3OC_{12}$-HSL) and *RhlI* guides the synthesis of butyryl -HSL (C_4-HSL) [8]. Consequently, functional QS necessitates transcription of the autoinducer synthase (*lasI, rhlI*) and response regulator (*lasR, rhlR*) and autoinducers production.

In the *las* system, the AHL signal molecule ($3OC_{12}$-HSL) triggers a transcriptional activator, *LasR*, to induce virulence factors expression such as elastase and toxin A [6]. The signal molecule ($3OC_{12}$-HSL) has also been found to be mandatory for differentiation of microcolony in Pseudomonas biofilm formation [9]. In the second QS system, *rhl*, the AHL signal (C_4-HSL) binds and activates the transcriptional protein RhlR to regulate the production of pyocyanin and elastase, in addition to haemolysin and alkaline protease [7]. These systems work in a categorized fashion together with another described quinolone signalling system [10].

Pseudomonas quinolone signal (PQS) system is an extra QS regulatory pathway in *P. aeruginosa* [10]. In vitro, *P. aeruginosa* QS systems has a hierarchical arrangement, with the *las* system on uppermost, regulating the *rhl* system. The PQS system is functionally located between the *las* and *rhl* systems. Nevertheless, it has been postulated that the *rhl* system can be activated independently of the *las* system, and it has been proposed that PQS system controls this activation [11].

QS in *P. aeruginosa* is one of the regulatory mechanisms of many virulence factors expression that is supposed to play a vital role in the pathogenicity of this organism [12].

Biofilm forming ability of *P. aeruginosa* plays a major role in development of Persistent infection with significant donations from discrete virulence factors as elastase [13], LPS [14], rhamnolipids [15] and alginate [16].

Biofilm formation has been found to be associated with QS in *P. aeruginosa.* Investigation of mutant strains of *P. aeruginosa* that are deficient in the production of the las signal molecule ($3OC_{12}$-HSL) revealed that the biofilm produced is more thinner moreover it does not display the three-dimensional architecture detected in the parent [12].

Limited number of in vivo researches in certain models have revealed that *P. aeruginosa* virulence is related to QS. Although QS- deficient mutants, predominantly strains deficient in *lasI, rhlI,* double *lasI/rhlI*, or *lasR* have been described to be less virulent in rat models of pneumonia[17], burn infection [18], in corneal infection[19], besides in a mouse model of chronic lung infection[20]. The starring role of QS systems in the virulence of *P. aeruginosa*- related implants associated infections remains unclear. Based on previous observations, we intended to survey some of QS-dependent virulence factors in *P. aeruginosa* isolates from retrieved different orthopedic implants.

METHODOLOGY

Study design:

A cross sectional descriptive study was conducted in the period from May 2015 to April 2017. Three hundred different orthopedic implants (plates, screws, intramedullary nails, wires and pins) and prostheses (knee / hip prostheses) retrieved from patients who were diagnosed clinically as orthopedic implant related osteoarticular infection, in the department of Orthopedic Surgery at Mansoura University Hospital were subjected to a sonication protocol as described by ***Vergidis et al.*** [21]. Fifty milliliters of the sonicate fluid were 100-fold concentrated by centrifugation at 2,000 x g for 20 min and resuspended, then 0.1 mL aliquots of the concentrated sonicate fluid were plated onto blood agar and macConkey's agar, incubated at 37°C for 24 hours. The protocol of this research was approved by the ethical committee in the Faculty of Medicine, Mansoura University.

Bacterial isolates:

Twenty-four isolates of *P. aeruginosa* were obtained from sonicate fluid. They were identified according to laboratory biochemical standards [22], and confirmed by API 20NE.

The isolates were cultured on Luria-Bertani (LB) agar and stocked in 20% glycerol/ LB broth at -80° C. These stock cultures were utilized in inoculation of media for different assays to evade repeated sub culturing of the *P. aeruginosa* isolates.

Preparation of supernatant of P. aeruginosa isolates:

An overnight culture of *P. aeruginosa* (0.5 ml) was inoculated into 4.5 ml of LB broth medium for 16-18 h at 37°C with shaking at 150 rpm. The supernatant was separated from the bacterial cells by centrifugation at 10.000 rpm for 15 mins at 4°C, and filtered. Cell free filtrates were kept in screw capped bottle at -20° C and utilized for the assay of QS autoinducers and some virulence factors [23].

P. aeruginosa PAO1 (the wild-type strain) was used as a positive control strain for phenotypic tests and for QS genes detection by PCR. *P. aeruginosa* PAO-JP2 (Δ*lasI*, Δ*rhlI* mutant), *P. aeruginosa* PAO-JP3 (Δ*lasR*, Δ*rhlR* mutant) were utilized as negative control strains for phenotypic tests. They were propagated under the same conditions.

Phenotypic detection of virulence factors

Pyocyanin assay:

King A media (peptone 2%, K2SO4 1%, and MgCl2 0.14%) was inoculated with *P. aeruginosa* supernatant and incubated at 37°C for 48h. The supernatant (5 ml) was mixed with chloroform (5 ml) then 0.2 M HCl (1.5 ml) was added to the separated lower organic layer, at that point, the organic layer rich in pyocyanin was separated. Pyocyanin was measured at the optical density OD520 nm. The concentration (µg/ml) was calculated according to the (OD 520 nm x 17.072) formula [24]. The taken value was the mean of three readings for each isolate.

Rhamnolipids assay

Cetyltrimethylammonium bromide (CTAB) agar test was done by inoculating an overnight (LB) broth culture of *P. aeruginosa* isolates (2 ml) on M9-glutamate minimal medium agar plates with CTAB (0.2 g) and methylene blue (5 mg). It was incubated at 37°C without shaking overnight then at room temperature for 48 hours followed by two days at refrigerator. CTAB precipitated in presence of methylene blue forming dark blue halo around *P. aeruginosa* growth on a light blue plate background that give an indication for rhamnolipid production [25].

LasB elastase assay

Ten mg/ml Elastin Congo Red (ECR) (Sigma, USA) was used to assess the elastolytic (LasB) activity within the *P. aeruginosa* isolates [26]. Ten mg of ECR and 0.5 ml of ECR buffer (100 mM Trise HCl, pH 7.5) were mixed with 0.5 ml supernatant fraction of *P. aeruginosa* isolate, and incubated for 6 hours at 37°C with shaking. OD495 nm was measured after centrifugation to eliminate insoluble ECR.

Protease assay

Modified skimmed milk assay technique was used to assess total proteolytic activity [27]. Culture supernatants of *P. aeruginosa* isolates (0.5 ml) were incubated with 1 ml skimmed milk (1.25%) at 37°C for 0.5 hour. The degree of clearance of skimmed milk was determined by measuring the turbidity at OD600 nm.

Biofilm Formation:

Microtiter plate assay was used to assess biofilm formation according to ***Stepanovic et al.***[28]. Briefly, cells from overnight culture on trypticase soy agar supplemented with 0.25% glucose were suspended in trypticase soy broth + 0.25 % glucose and adjusted at OD600. One hundred µL of the suspension was poured into the individual wells of sterile flat-bottomed 96-well polystyrene tissue culture plates (Sigma-Aldrich, USA), at least in triplicate. The plates were incubated for 24 hours at 37°C without shaking. After incubation, contents of each well were removed by gentle tapping, and wells were 3 times washed with sterile phosphate-buffered saline (PBS; pH 7.2). The fixation step was done by air drying and the adherent biofilm layer was stained by 0.1 % crystal violet solution (20 min). This was followed by the washing steps. Then the plates were air dried and resolubilized with ethanol (95%) for 30 minutes. Finally, OD of individual well was measured 492 nm using a microtiter plate reader. For each strain, the mean OD of the three wells was calculated (ODT). OD cut-off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control.

Autoinducer (AHLs) detection by Cross-feeding bioassay:

Autoinducer (AHLs) was detected on LB plates using C. violaceum CV026 and the C. violaceum VIR07 as reporter strains, which respond to C_4-HSL as well as $3OC_{12}$-HSL respectively by producing the purple pigment violacein [29].

PCR for detection of *lasI, lasR, rhlI, rhlR* genes:

DNA was extracted from tested *P. aeruginosa* isolates and from *P. aeruginosa* reference strain PAO1. One colony of each strain cultured on solid medium was inoculated into 5 ml of LB broth. After overnight incubation at 37°C; DNA was extracted from these cultures utilizing DNA extraction mini kit (QIAgen, Hilden, Germany) according to the manufacturer's instructions.

PCR was performed in a 25-µL reaction mixture containing a half volume of PCR master mix (BioMix Red; Luckenwalde, Germany), 2 µL of bacterial DNA template and 100 pmol of each primer [30] (Table 1). Thirty cycles of amplification were performed at 37°C according to the following Parameters; denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute, and primer extension at 72°C for 1.5 minutes. Amplified products were detected on (2%) agarose gel electrophoresis.

Table 1: Specific amplification primer sets for the tested Quorum sensing genes among *P. aeruginosa* isolates

gene	*type*	*sequence*	*Amplicon size (bp)*
lasI	F	5′-CGCACATCTGGGAACTCA-3′	176
	R	5′-CGGCACGGATCATCATCT-3′	
LasR	F	5′-CTGTGGATGCTCAAGGACTAC-3′	133
	R	5′-AACTGGTCTTGCCGATGG-3′	
rhlI	F	5′-GTAGCGGGTTTGCGGATG-3′	101
	R	5′-CGGCATCAGGTCTTCATCG-3′	
rhlR	F	5′-GCCAGCGTCTTGTTCGG-3′	160
	R	5′-CGGTCTGCCTGAGCCATC-3′	

RESULTS

QS-dependent virulence factors as pyocyanin, Rhamnolipid, las B elastase, protease, and biofilm formation were assayed in studied *P. aeruginosa* isolates (n = 24) and in PAO1, PAO-JP2, and PAO-JP3 strains.

Seventeen (70.8%) isolates displayed the QS-dependent phenotypes and seven isolates were found to be lacking all tested virulence factors. The lack of production of pyocyanin, Rhamnolipid, las B elastase, protease, and biofilm formation by the seven isolates proposed that they may have a defective QS system.

Nine (37.5 %) isolates produced less elastase than PAO-JP3 (*lasR* mutant), 15 (62.5 %) isolates produced elastase in a level between those of PAO-JP3 and PAO1. Fourteen (58.3%) isolates were positive producers for pyocyanin and protease, while 10 (41.7%) isolates were negative for both. Thirteen (54.2%) isolates were capable of rhamnolipids production, while eleven (45.8%) isolates were non-producers. sixteen (66.7 %) isolates were biofilm producers, though eight (33.3%) isolates were non biofilm producers in comparison with PAO1 strain.

Seven isolates (3, 8, 13, 14, 18, 21, 23) exhibiting impaired QS-dependent phenotypes (Table 2) were additionally analysed for AHLs ($3OC_{12}$-HSL and C_4-HSL) production by cross-feeding bioassay, and for presence of QS genes (*lasI, lasR, rhlI, rhlR*) by PCR.

AHLs autoinducers were present in three isolates (3, 18, 21), whereas the remaining four isolates lacked production of both autoinducers and thus considered QS-deficient.

The failure of production of C_4-HSL and $3OC_{12}$-HSL and virulence factors by four isolates (8, 13, 14, and 23) might be because of the loss of any one of the QS system genes. Along these lines, we checked this probability by trying to amplify *lasI, lasR, rhlI* and *rhlR* genes by PCR and used PAO1 as the positive control. PCR analysis revealed that two isolates (8, 14) possessed *lasI, lasR, rhlI* and *rhlR* genes while one isolate (13) lacked *lasR* gene, and one isolate (23) was deficient in all four genes tested (Table 2).

Table (2): Virulence factors, Quorum sensing genes, and autoinducer production in QS deficient *Pseudomonas aeruginosa* isolates (n=7)

Isolate number	*Virulence Factors**					*Quorum sensing genes*				*Autoinducer*	
	Pyocyanin	**Rhamnolipids**	**LasB elastase**	**Total protease**	**Biofilm**	***lasI***	***lasR***	***rhlI***	***rhlR***	**$3OC_{12}$-HSL**	**C_4-HSL**
3	28	-	16	6	20	+	+	+	+	+	+
8	13	-	19	3	17	+	+	+	+	-	-
13	5	-	23	24	11	+	-	+	+	-	-
14	18	-	0	21	37	+	+	+	+	-	-
18	37	-	15	12	42	+	+	+	+	+	+
21	1	-	13	16	15	+	+	+	+	+	+
23	3	-	12	27	1	-	-	-	-	-	-
PAO 1	100	+	100	100	100	+	+	+	+	+	+

* Activity percentages as regard PAO1 (positive control strain) activity that was considered 100%

DISCUSSION

Our study was targeted to assess some QS-dependent virulence factors in *P. aeruginosa* isolates from retrieved different orthopedic implants.

Twenty-four *P. aeruginosa* isolates from 300 retrieved different orthopedic implants were assessed for f QS-dependent virulence factors.

Among the studied 24 *P. aeruginosa* isolates, 70.8% (17/24) were found to display the QS-dependent phenotypes. 62.5% produced elastase, 58.3% produced both pyocyanin and protease, 54.2% produced rhamnolipid, and 66.7% were biofilm producers, proposing that these isolates were QS proficient. This study draws on previous researches conducted and approves the fundamental role of QS in virulence of *P. aeruginosa* [31,32].

We identified seven isolates exhibiting impaired QS-dependent phenotypes i.e. lacked the production of all tested virulence factors. These isolates were additionally analyzed for AHLs ($3OC_{12}$-HSL and C_4-HSL) production by cross-feeding bioassay. Both AHLs autoinducers were confirmed to be present in three isolates. While, the residual four isolates lacked production of both and hence considered QS-deficient.

While the assay for both autoinducers production and tested QS (*lasI, lasR, rhlI* and *rhlR)* genes were confirmed in three of the tested isolates (3, 18, and 21),

they were abortive to display the QS-dependent phenotypes. Even though this finding was approved in vitro, these strains might have extra virulence in vivo. Our results provide an evidence that there may be additional uncharacterized factors rather than the well-known virulence factors that is involved in *P. aeruginosa* pathogenesis.

Many other global regulators other than QS *lasI/R and rhlI/R* are involved in the regulation of QS systems. Numerous extra regulatory proteins have been revealed to control the *las* or *rhl* system, as QscR [33], RpoS [34], GacA [35], and Vfr [36]. Additional signal molecule, 2-heptyl- 3-hydroxy-4-quinolone, denoted as PQS, has been recognized as an inducer for both *lasB* and *rhlI* in *P. aeruginosa* [10]. The activity and production of this signal was also reliant on *RhlR* and *LasR* [10].

Molecular study of QS-genes in these isolates revealed that 2 isolates (8, 14) contained *lasI, lasR, rhlI* and *rhlR* genes while in one isolate (13) *lasR* gene was not detected, and in one isolate (23) all QS genes (*lasI, lasR, rhlI, rhlR*) failed to be amplified with the primers we used.

Similarly, in a study conducted by ***Schaber et al.*** [37], they recognized an isolate of *P. aeruginosa* with no pyocyanin or elastase activity, which lacks both autoinducers production although having all QS genes.

The explanation of QS-deficient phenotype of isolates 13 and 23 might be elucidated by defects of *lasR* and *lasI, lasR, rhlI* and *rhlR* genes respectively.

Failure of amplification by PCR does not argue lack of QS genes as absence or mutation of the target sequences may lead to absence of PCR product. Contrariwise, existence of amplified product does not ignore the likelihood that the QS genes may have had disabling mutations and this might clarify the defective QS-dependent phenotypes noticed in the five isolates in which all tested QS (*lasI, lasR, rhlI* and *rhlR*) genes were amplified with PCR.

Few studies [37,38] reported the effect of autoinducers (C_4-HSL and $3OC_{12}$-HSL) signaling molecules loss and lack of several virulence factors on *P. aeruginosa* clinical isolates pathogenesis. In a study conducted by ***Senturk et al.*** [39], they reported that QS deficient clinical isolates that were incapable of production of C_4-HSL signaling molecule and its dependent virulence factors because of mutations in the *rhlR* and /or *RhlI* genes however they are able to cause infections in humans. In addition, ***Schaber et al.*** [37] reported one QS deficient clinical isolate with absence of all tested virulence factors, nevertheless still caused a wound infection.

In a study conducted by ***Bosgelmez-Tinaz and Ulusoy*** [40], they reported that despite obvious defect in *rhl* system, their isolates had a functional *las* QS system and produced the $3OC_{12}$-HSL signaling molecule. Many other clinical studies revealed that any single virulence factor loss would be recompensed by other virulence factors through infection [18].

Senturk et al. [39] suggested that the existence of multiple *P. aeruginosa* strains at the infection site may prime a QS deficient strain to cause infection. Signaling molecules and/or QS-regulated factors production by QS proficient strains may assist a QS deficient strain to cause infection. If the patient infected by two *P. aeruginosa* strains; one is QS proficient and the other is QS deficient, the QS deficient strains might revenue from products of QS proficient one as the extracellular enzymes.

Even though virulence factors and biofilm formation in *P. aeruginosa* seems to be controlled in a cell density–dependent fashion through QS, our results confirms that *P. aeruginosa* is capable of producing implant infection in patients despite an impaired QS system. These results do not challenge the concept that QS plays a foremost role in pathogenicity of *P. aeruginosa*, but highlight that along with known virulence factors, perhaps there are additional virulence factors, that may not be strictly under QS control. This also support the concept that *P. aeruginosa* virulence is multifactorial.

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