

Quorum sensing and virulence of *Pseudomonas aeruginosa* during urinary tract infections

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Abstract

Introduction: In the opportunistic pathogen *Pseudomonas aeruginosa*, the production of several virulence factors depends on quorum sensing (QS) involving *N*-acylhomoserine lactone signal molecules. *In vitro* studies have suggested that the QS system is crucial in the pathogenesis of *P. aeruginosa*. However, it is unclear whether QS systems of *P. aeruginosa* play the same role during infections.

Methodology: In this study, to explore the contribution of QS systems to the pathogenesis of *P. aeruginosa* during urinary tract infections, we collected 82 clinical isolates. Detection of *N*-acyl-homoserine lactones (C12-HSL and C4-HSL) was performed on agar plates employing biosensor strains *C. violaceum*. Elastase and biofilm production were determined spectrophotometrically. QS genes were detected by PCR and subsequently underwent sequencing.

Results and conclusion: Six isolates were found to be negative in the production of both C12-HSL and C4-HSL and all virulence factors tested. PCR analysis of these isolates revealed that four isolates contained all four QS genes while one isolate was negative for *lasR* gene, and one isolate negative for *lasI*, *lasR* and *rhlR* genes. Sequence analyses of these isolates showed that the *lasR*, *lasI*, *rhlR* and *rhlI* genes had point mutations. The combination of these mutations probably explains their C12-HSL, C4-HSL and virulence factor deficiencies. Results of this study suggest that QS deficient clinical isolates occur and are still capable of causing clinical infections in humans.

Key words: *P. aeruginosa*; quorum sensing; virulence factors

J Infect Dev Ctries 2012; 6(6):501-507.

(Received 19 January 2012 – Accepted 23 March 2012)

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Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen that preferentially infects patients with cancer or AIDS, patients immunocompromised by surgery, cytotoxic drugs or burn wounds, people with cystic fibrosis or blood, skin, eye and genitourinary tract infections [1,2]. It can also colonize implanted devices, catheters, heart valves or dental implants [3,4]. One of the reasons that *P. aeruginosa* is a successful opportunistic pathogen is that it produces an array of virulence factors, including elastases (LasB and LasA), alkaline protease, pyocyanin and rhamnolipids [5]. The genes encoding these virulence factors are often controlled in a cell-dependent fashion through a mechanism known as quorum sensing (QS).

P. aeruginosa possesses two QS systems, *las* and *rhl*, which are regulated by *N*-acyl-homoserine lactones (AHLs). The *las* system comprises LasI, which is responsible for the synthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-

HSL), and the transcriptional activator LasR [6, 7]. This system was shown to regulate the production of elastase, exotoxinA and alkaline protease. The *rhl* system, relying on *N*-butanoyl-L-homoserine lactone (C4-HSL), was shown to be involved in the regulation of rhamnolipid, alkaline protease, elastase, cyanide and pyocyanin production [8, 9]. The two QS systems of *P. aeruginosa* are hierarchically linked. The *las* system positively regulates the expression of both *rhlR* and *rhlI* [6,10,11]. An additional signalling molecule, 2-heptyl-3-hydroxy-4-quinolone (PQS), has been identified. The production and activity of PQS was shown to be dependent on LasR and RhlR [12]. The PQS also appears to regulate *rhlI* expression [13]. In addition, global regulators such as Vfr, GacA, RpoS and RpoN have also been demonstrated to regulate the expression of virulence factors [14-16].

The importance of QS in the virulence of *P. aeruginosa* has been shown in various animal studies in models of burn wound infection and pneumonia

[15,17-19]. These studies indicate that the QS systems of *P. aeruginosa* play an important role during respiratory tract infections and burn wound infections in the mouse model. However, only a few studies are available concerning the role of *P. aeruginosa* QS systems in urinary tract infections.

To extend our understanding of the role of QS in *P. aeruginosa* in the establishment of urinary tract infection in humans, we collected 82 clinical isolates from patients of Marmara University Hospital. Isolates were screened for the production of C12-HSL and C4-HSL QS signaling molecules and virulence factors (elastase, rhamnolipid) along with their biofilm formation capacities.

Methodology

Bacterial strains, media and growth conditions

Eighty-two non repeat clinical isolates of *P. aeruginosa* were obtained from patients of Marmara University Hospital. The isolates were collected within a period of three years (2007-2010) and confirmed to be *P. aeruginosa* by Phoenix (Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) and Vitek 2 (Bio-Mérieux, I'Etoile, France) automated identification systems. *P. aeruginosa* strains were routinely grown in Luria-Bertani (LB) broth at 37 °C [20]. *C. violaceum* CV026 and *C. violaceum* VIR07 biosensor strains were routinely grown in or on LB (1% tryptone, 0.5% yeast extract, 0.5 % NaCl) solidified with 1.2% agar when required and supplemented with kanamycin (20 µg/ml). Standard laboratory reference strains, the wild-type strain *P. aeruginosa* PAO1, *P. aeruginosa* PAO-JP2 ($\Delta lasI$, $\Delta rhII$ mutant) and *P. aeruginosa* PAO-JP3 ($\Delta lasR$, $\Delta rhIR$ mutant) were used as controls in phenotypic and genotypic tests.

Cross-feeding bioassay for detection of AHLs

Detection of AHLs was determined on agar plates employing biosensor strains *C. violaceum* CV026 and the *C. violaceum* VIR07, which respond to short chain and long chain AHLs respectively by producing the purple pigment violacein [21,22].

Elastase assay

Elastase activity was measured using the elastin Congo red (ECR; Sigma) assay [23]. Cells were grown in LB broth at 37°C for 16 hours, centrifuged at 15 000g at 4°C for 10 minutes and 0.5 mL supernatant was added to 1 mL of assay buffer (30 mM Tris buffer, pH 7.2) containing 10 mg of elastin Congo Red. The mixture was incubated at 37°C for 6

hours. Insoluble ECR was removed by centrifugation, and the absorption of the supernatant was measured at 495 nm. LB medium was used as a negative control.

Production of rhamnolipids

M9-glutamate minimal medium agar plates containing 0.2 g cetyltrimethylammoniumbromide (CTAB) and 5mg methylene blue l⁻¹ were inoculated with 2 ml of an overnight LB culture of *P. aeruginosa* strains. After an overnight incubation at 37°C, the diameter of the clearing zone around the bacterial spots was measured as evidence of rhamnolipid production [24].

Biofilm assay

Bacterial biofilms were developed at 35°C in 96-well microtiter plates containing LB medium. After inoculation, microtiter plates were incubated at 35°C for 8 hours and then washed to remove planktonic bacteria. The plates were stained with 0.1% crystal violet for ten minutes. After staining, plates were washed with sterile distilled water three times to remove crystal violet solution. The quantitative analysis of biofilm production was performed by adding 200 µl of 95% ethanol to destain the wells. Then 125 µl of the crystal violet/ethanol solution was transferred to a new microtiter plate and optical density (OD) of the samples was measured at 550 nm [25].

PCR for detection of the quorum-sensing genes

Chromosomal DNA was extracted using a commercial kit (High Pure PCR Template Kit, Roche Diagnostics, Basel, Switzerland). Oligonucleotide primers *lasR1* (5'-atggccttggtgacggtt-3') and *lasR2* (5'-gcaagatcagagagtaataagaccca-3'), *lasI1* (5'-atgatcgtacaaattggtcggc-3') and *lasI2* (5'-gtcatgaaaccgccagtcg-3'), *rhlR1* (5'-caatgaggaatgacggaggc-3') and *rhlR2* (5'-gcttcagatgaggcccagc-3'), *rhlI1* (5'-cttggtcatgatgaattgctc-3') and *rhlI2* (5'-acgctgacgacctcacac-3') were used to amplify *lasR*, *lasI*, *rhlR* and *rhlI* genes, respectively (Schaber JA, 2004). PCR was performed in 50 µl of reaction mixture containing 30 ng of chromosomal DNA, 25 µl master mix (Fermentas), 100 pmol (1.5 µl) of each primers. PCR conditions for the amplification step were as follows: initial denaturation at 94°C for 30 seconds followed by 34 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 2 minutes, and a final extension at 72°C for 10 minutes.

Table 1. Summary of production of virulence factors, C12-HSL and C4-HSL signaling molecules and the presence of OS genes of OS deficient *P. aeruginosa* clinical isolates

Strain	Virulence factor		Rhamnolipid	Signaling Molecule		Quorum Sensing Genes			
	Elastase Activity*	Biofilm Formation*		C12-HSL	C4-HSL	<i>lasI</i>	<i>lasR</i>	<i>rhlI</i>	<i>rhlR</i>
1	13	34	-	-	-	+	+	+	+
5	13	37	-	-	-	+	-	+	+
49	13	39	-	-	-	+	+	+	+
60	11	16	-	-	-	+	+	+	+
67	12	15	-	-	-	-	-	+	-
78	14	27	-	-	-	+	+	+	+
PAO 1	100	100	+	+	+	+	+	+	+
PAO	14	35	-	-	-	-	+	-	+
PAO	14	39	-	-	-	+	-	+	-

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

Sequencing of *lasR*, *lasI*, *rhlR* and *rhlI* genes

To search for the presence of mutations that may affect the QS genes, *lasI*, *lasR*, *rhlR* and *rhlI* underwent sequencing. The PCR products were purified by using the Qiaquick PCR purification kit (Qiagen, Courtaboeuf, France) and then quantified following electrophoresis on agarose gels by using a marker of known quantity (Fermentas, 1 kb DNA ladder, Vilnius, Lithuania). Automated sequencing reactions were performed with the BigDye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer by IONTEK Company (Istanbul, Turkey) using the same primers that were used in amplification of the QS genes. The non-primer-derived sequences were aligned and compared with those of the published PAO1 strain of *P. aeruginosa* using the National Center for Biotechnology Information BLAST server.

Results

Production of AHLs dependent virulence factors

To determine the contribution of QS systems to the pathogenesis of *P. aeruginosa* during urinary tract infections, 82 isolates were obtained from patients with urinary tract infections at Marmara University Hospital. All isolates were screened for the production of AHLs dependent virulence factors, elastase, rhamnolipid and biofilm formation.

The production of elastase by the isolates was examined by the elastin Congo red assay. *In vitro* levels of elastase produced by the 82 clinical isolates were compared with those produced by the *lasR* mutant strain (PAO-JP3) and QS wild-type strain

(PAO1). Eleven (13.4 %) isolates produced less elastase than the *lasR* mutant PAO-JP3, 6 (7.3 %) isolates produced higher levels of elastase than PAO1, and 65 (79.2 %) isolates produced levels of elastase between those of PAO-JP3 and PAO1 (Figure 1). Fifty-three (64.6%) of the isolates were able to produce rhamnolipids while no rhamnolipid production was observed in 29 (35.4%) isolates. Sixty-four (78 %) isolates produced biofilm whereas 18 (22%) of the isolates formed almost no biofilm compared to strain PAO1 (Fig 2).

The screening for the production of virulence factors in 82 clinical isolates revealed that six isolates (1, 5, 49, 60, 67 and 78) were deficient in all virulence factors tested (Table 1). The lack of elastase and rhamnolipid production and biofilm formation by the six isolates suggested that these isolates might have a defective QS system.

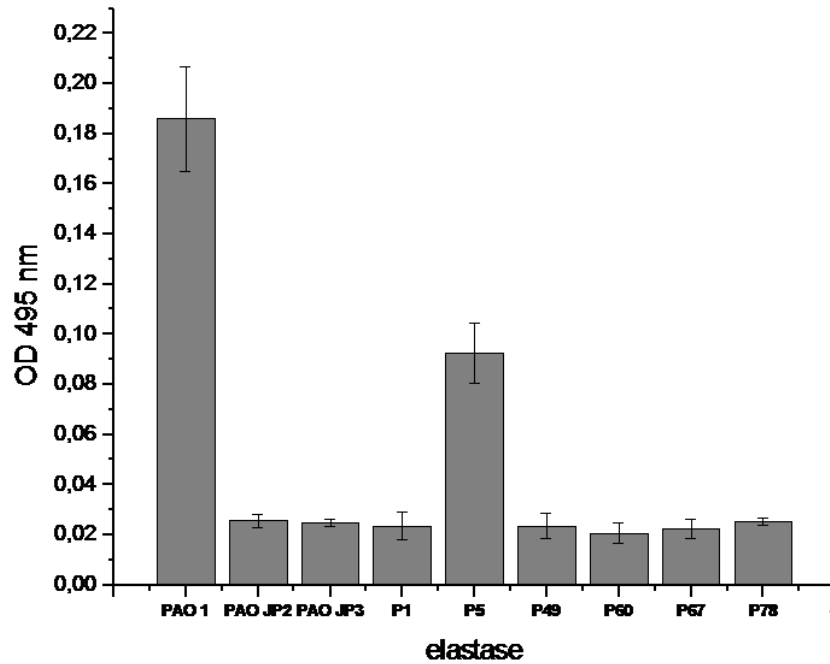
Detection of AHL molecules

Detection of AHL molecules produced by six isolates was determined using a cross-feeding bioassay as described in the Methodology section. Isolates 1, 5, 49, 60, 67 and 78 were cross-streaked with *C. violaceum* CV026 and the *C. violaceum* VIR07. Results of this assay suggested that isolates 1, 5, 49, 60, 67 and 78 were defective in the production of both C12-HSL and C4-HSL (Table 1).

PCR detection of quorum sensing genes

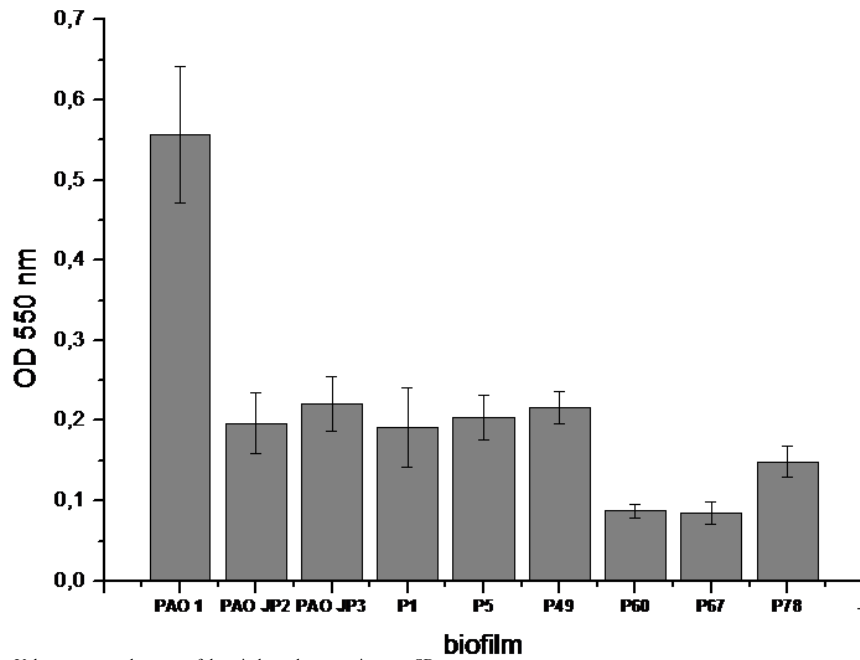
The failure of six isolates to produce C4-HSL and C12-HSL and virulence factors may be due to the loss of any one of the QS system genes. We therefore

Figure 1. Elastase assay of the culture supernatants of the six QS deficient clinical isolate.



PAO1 was used as positive control while PAO-JP2 and PAO-JP3 served as negative controls. Values represent the mean of three independent experiments \pm SD.

Figure 2. Biofilm formation capacities of QS deficient isolates. PAO1 was used as positive control while PAO-JP2 and PAO-JP3 served as negative controls.



Values represent the mean of three independent experiments \pm SD.

checked this possibility by attempting to amplify intact *lasR*, *lasI*, *rhlI* and *rhlR* genes by PCR. Oligonucleotide primers used for the PCR experiments are given in the Methodology section. PA01 was used as the positive control. PCR analysis revealed that four isolates (1, 49, 60 and 78) contained *lasR*, *lasI*, *rhlR* and *rhlI* genes while one isolate was negative for *lasR* gene (5), and one isolate (67) was negative for *lasI*, *lasR* and *rhlR* genes (Table 1).

Isolates 1, 49, 60, 78 contained all *lasR*, *lasI*, *rhlR* and *rhlI* genes but were deficient in both autoinducers, and virulence factors were further investigated for mutational defects in these QS genes.

Sequencing analyses of *lasR*, *lasI*, *rhlI* and *rhlR* genes

To determine if QS genes have mutations, we sequenced *lasR*, *lasI*, *rhlR* and *rhlI* genes of isolates 1, 49, 60 and 78. Sequence analysis of the PCR products showed that these four isolates appeared to carry various mutations in the QS genes. Isolates 1 and 49 had mutational defects in the *lasR*, *rhlR* and *rhlI* genes while isolate 60 had mutations in the *lasR* and *rhlR* genes and isolate 78 had defects in the *rhlI* and *rhlR* genes. None of the isolates had any mutations in the *lasI* gene. This could probably explain their virulence factor-negative phenotype, as well as their deficiency in AHL production.

Discussion

In the present study, we examined the role of QS in the pathogenesis of *P. aeruginosa* in urinary tract infections. We measured the production of autoinducers C12-HSL and C4-HSL and three QS-dependent virulence factors in 82 *P. aeruginosa* urinary tract isolates. A total of 86.58% of these isolates produced elastase, 64.6% produced rhamnolipids, and 78% produced biofilm, suggesting that these isolates were QS proficient. This observation agrees with the previous studies and confirms the crucial role of QS in *P. aeruginosa* virulence [26-31]. 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 [32-34]. In these studies, inadequacy of QS deficient strains to establish successful infection was proposed to be associated with reduced production of virulence factors.

However, in the present study, among the 82 isolates we identified 6 isolates that were defective in production of all virulence factors tested, as well as two AHLs (C12-HSL and C4-HSL), but these isolates still cause urinary tract infections in humans. PCR analysis of these isolates for the presence of QS-genes revealed that four isolates (1, 49, 60 and 78) contained *lasR*, *lasI*, *rhlR* and *rhlI* genes while one isolate was negative for *lasR* gene (5), and one isolate (67) was negative *lasI*, *lasR* and *rhlR* genes. The QS-deficient phenotype of isolates 5 and 67 could be explained by the loss of *lasR* and *lasI*, *lasR* and *rhlR* genes respectively. Sequence analysis of four strains which contained all QS genes (1, 49, 60 and 78) revealed that these had several mutations in the *lasR*, *lasI*, *rhlR* and *rhlI* genes, probably explaining the defect in both autoinducers C12-HSL and C4-HSL and virulence factor production. In our previous study, we reported QS deficient clinical isolates that were unable to produce the C4-HSL signaling molecule and C4-HSL dependent virulence factors as a result of mutations in the *rhlR* and/or *rhlI* genes but still cause infections in humans. Despite this apparent deficiency in their *rhl* system, these isolates had a functional *las* QS system and produced the C12-HSL signaling molecule [35]. Several other clinical studies also showed that the loss of any single virulence factor appeared to be compensated by other virulence factors during infection [29,36]. However the impact of the loss of both C4-HSL and C12-HSL signaling molecules and several virulence factors on the pathogenesis of *P. aeruginosa* clinical isolates has been reported in very few studies [30,37]. Schaber *et al.* [30] identified one QS deficient clinical isolate which lost all virulence factors tested, yet still caused a wound infection. Similar to our findings, Dénervaud *et al.*, [37] identified three *P. aeruginosa* strains, obtained from intubated patients, 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*. Our results provide additional evidence supporting this hypothesis.

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.

In conclusion, the results of this study confirm that the QS systems play an important role in the pathogenesis of *P. aeruginosa* infections of the urinary tract and indicate that *P. aeruginosa* is capable of causing clinical infections in humans despite an impaired QS system. These findings do not contradict the theory that QS plays a major role in *P. aeruginosa* pathogenicity, but emphasise that in addition to known virulence factors, there may be other virulence factors which may not be stringently controlled by QS.

Acknowledgements

We thank Tomohiro Morohoshi for providing *C. violaceum* VIR07. This work was supported by a project grant (SAG-C-YLP-010710-0215) from the Marmara University Scientific Research Fund.

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Conflict of interests: No conflict of interests is declared.