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Analysis of quorum sensing-dependent virulence factor production and its relationship with antimicrobial susceptibility in *Pseudomonas aeruginosa* respiratory isolates

O. Karatuna and A. Yagci

Department of Microbiology, School of Medicine, Marmara University, Haydarpasa, Istanbul, Turkey

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen causing severe respiratory infections. The pathogenesis of these infections is multifactorial and the production of many virulence factors is regulated by quorum sensing (QS), a cell-to-cell communication mechanism. The two well defined QS systems in *P. aeruginosa*, the *las* and *rhl* systems, rely on *N*-acyl homoserine lactone signal molecules, also termed autoinducers. We assessed the activity of QS-dependent virulence factors (including elastase, alkaline protease, pyocyanin and biofilm production) in respiratory isolates of *P. aeruginosa* and their relationship with antimicrobial susceptibility. We identified sixteen isolates displaying impaired phenotypic activity; among them, eleven isolates were also defective in autoinducer production, and therefore considered QS-deficient. Six of the QS-deficient isolates failed to amplify one or more of the four QS regulatory genes (*lasI*, *lasR*, *rhlI*, *rhlR*) with PCR: one isolate was negative for *rhlR*, two isolates were negative for *rhlI* and *rhlR* and three isolates were negative for all four genes. The isolates that were negative for virulence factor production were generally less susceptible to the antimicrobials and statistically significant correlations were observed between the lack of elastase production and resistance to piperacillin and ceftazidime; between failure in alkaline protease production and resistance to tobramycin, piperacillin, piperacillin-tazobactam, cefepime, imipenem and ciprofloxacin; and between failure in pyocyanin production and resistance to amikacin, tobramycin, ceftazidime, ciprofloxacin and ofloxacin. The results obtained indicate that, despite the pivotal role of QS in the pathogenesis of *P. aeruginosa* respiratory infections, QS-deficient strains are still capable of causing infections and tend to be less susceptible to antimicrobials.

Keywords: Antimicrobial susceptibility, *Pseudomonas aeruginosa*, quorum sensing, virulence
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Corresponding author: A. Yagci, Marmara University, School of Medicine, Department of Microbiology, 34668, Haydarpasa, Istanbul, Turkey
E-mail: ayagci@marmara.edu.tr

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen with remarkable metabolic versatility, which allows it to thrive in diverse natural and nosocomial conditions. Infections as a result of *P. aeruginosa* are rare in otherwise healthy individuals but common in the compromised, such as patients with cystic fibrosis (CF), severe burns, or those with impaired immunity as seen in patients infected with HIV or in cancer patients undergoing chemotherapy [1]. The respiratory system is the most frequent body site infected, often presenting as acute ventilator-associated pneumonia in nosocomial settings or chronic destructive lung disease in patients suffering from CF [2]. The pathogenesis of *P. aeruginosa* lung infections is multifactorial and depends on numerous virulence factors, including the secreted factors such as elastase, alkaline protease, phospholipase C, exotoxin A, exoenzyme S, pyocyanin, pyoverdine, hydrogen cyanide, rhamnolipid and cell-associated factors, such as lipopolysaccharide, flagella and pili [1,3,4]. Another factor contributing to pathogenesis of *P. aeruginosa* is its tendancy to form organized communities, known as biofilms when it attaches to biotic or abiotic surfaces.

In recent years, it has been discovered that the production of many virulence factors and the formation of biofilm by *P. aeruginosa* is regulated by a cell-to-cell communication mechanism known as quorum sensing (QS) [5,6]. The two

well-defined QS systems in *P. aeruginosa*, namely *las* and *rhl*, rely on *N*-acyl homoserine lactone (AHL) signal molecules, also termed autoinducers. The *las* system is comprised of the transcriptional regulatory protein LasR, its cognate autoinducer molecule *N*-(3-oxododecanoyl) homoserine lactone ($3OC_{12}$-HSL) and the AHL synthase LasI. Similarly, the *rhl* system consists of RhlR, its cognate autoinducer molecule *N*-butyryl homoserine lactone (C_4-HSL) and the AHL synthase RhlI [7]. When a critical threshold concentration of autoinducer is reached, the autoinducer molecule binds to its cognate transcriptional regulatory protein. The autoinducer-transcriptional regulatory protein complex in turn triggers the expression of many target genes, along with the AHL synthase, thus creating a positive feedback loop [6]. Although the *las* and *rhl* systems are two separate QS systems, they are related in a hierarchical manner, with the *las* system being dominant over the *rhl* system [8].

The essential role of QS in the pathogenesis of *P. aeruginosa* respiratory infections has been demonstrated in various animal models, although its importance in human lung infections remains speculative because QS-deficient isolates have been obtained from such infections [9–12]. In the present study, we aimed to assess the activity of QS-dependent virulence factors in clinically pathogenic respiratory isolates of *P. aeruginosa* and their relationship with antimicrobial susceptibility, another important virulence factor of *P. aeruginosa*.

Materials and Methods

Bacterial strains and growth conditions

The clinical *P. aeruginosa* isolates used in the present study were collected by the clinical microbiology laboratory at Marmara University, School of Medicine, Istanbul, Turkey, between the years 2005 and 2006. One hundred bacterial isolates obtained from lower respiratory clinical samples (sputum $n = 53$, tracheal aspirate $n = 37$, bronchoalveolar lavage $n = 10$) were presumptively identified as *P. aeruginosa* by biochemical tests and further identified to the species level using automated identification systems (Vitek 2, bioMérieux, l'Etoile, France; Phoenix, Becton-Dickinson, Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA). To discriminate between respiratory colonization and infection, the CDC guidelines were strictly followed and only clinical strains isolated from infections were included in the study [13]. To minimize clonal relatedness, only one isolate per patient was used in the study and patients were selected so that 35 of the isolates were from outpatients, 29 were from patients hospitalized in two different intensive care units (ICU) and 36 were from clinics other than ICU. The isolates were stored at −80°C in cryopreservation vials (Cryobank, Mast Diagnostics, Bootle, UK). Frozen stock cultures were used to inoculate the subcultures which were routinely grown in Luria Bertani (LB) broth (Difco, Franklin Lakes, NJ, USA) overnight at 37°C. Secondary media were inoculated as necessary for different assays. Secondary media included LB broth for elastin congo red, alkaline protease and biofilm assays (at 37°C for 16 h), LB agar (Difco) for micro-AHL cross-feeding bioassay. Glycerol alanine minimal medium (per litre: 10 mL glycerol, 6 g L-alanine, 2 g $MgSO_4$, 0.1 g K_2HPO_4, 0.018 g $FeSO_4$, sterilized in autoclave at 121°C for 15 min) was employed for pyocyanin assay (at 37°C for 24 h) [9]. *P. aeruginosa* PAO1 wild-type strain was used as positive control for phenotypic and genotypic tests. *P. aeruginosa* PAO-JP2 (Δ*lasI*, Δ*rhlI* mutant) and PAO-JP3 (Δ*lasR*, Δ*rhlR* mutant) strains were used as negative controls for phenotypic tests [14].

Elastin Congo red assay

The elastolytic activity of LasB elastase within the supernatant fraction of isolates was assayed using elastin congo red as substrate. Briefly, the cells were grown in LB broth at 37°C for 16 h, centrifuged at 15 000 ***g*** at 4°C for 10 min 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 (Sigma, Poole, UK). The mixture was incubated at 37°C for 6 h with constant rotation, insoluble substrate was pelleted with centrifugation at 1200 ***g*** for 10 min and absorbance of the supernatant was measured at 495 nm [15]. Assays were performed in triplicate in three different occasions.

Alkaline protease assay

Alkaline protease activity within the supernatant fraction of isolates grown overnight in LB broth was determined by adding 0.5 mL of supernatant to 1.5 mL of assay buffer (20 mM Tris-HCl, 1 mM $CaCl_2$ buffer, pH 8.0) containing 50 mg of hide remazol blue powder (Sigma). Tubes were incubated at 37°C for 1 h with constant rotation, reactions were stopped on ice, centrifuged at 4000 ***g*** for 5 min and absorbance of the supernatant was measured at 590 nm [16]. Assays were performed in triplicate in three different occasions.

Pyocyanin assay

Pyocyanin was extracted from broth cultures of isolates grown in glycerol alanine minimal medium for 24 h [10]. The cells were removed by centrifugation and pyocyanin in the supernatant was extracted into chloroform by mixing 5 mL of supernatant with 3 mL of chloroform. Pyocyanin was then reextracted into 1 mL of acidified water (0.2 M HCl) which

gave a pink–red solution. For the quantitation of the pyocyanin within the solution, the absorbance was measured at 520 nm [17]. Assays were performed in triplicate in three different occasions.

Biofilm assay

The biofilm forming ability of the isolates was tested using polystyrene microtitre plates. Briefly, overnight broth cultures in LB broth were diluted 1:100 into fresh LB broth and then 0.1 mL of the freshly inoculated medium was dispensed into wells of a 96-well polystyrene microtitre plate. The plate was incubated at 37°C for 8 h without agitation. Biofilms were detected by staining the wells with 10 μL of crystal violet [0.1% (w/v) in H_2O], after the stain was added the plate was incubated for another 15 min at room temperature and then washed thoroughly with distilled water to remove planktonic cells and residual dye. Ethanol (95%) was used to elute crystal violet from the biofilms and the absorbance of the solubilized dye was measured at 590 nm using a microtitre plate reader (Labsystems Multiskan MS) [18]. Assays were performed in triplicate on three different occasions.

Micro-AHL cross-feeding bioassay

To analyse AHL production, a previously presented quantitative microplate assay was used with modifications [19]. The assay relies on the induction of pigment production by the biosensor strains *Agrobacterium tumefaciens* A136 (most sensitive to $3OC_{12}$-HSL) and *Chryseobacterium violaceum* CV026 (most sensitive to C_4-HSL) subsequent to diffusion of the AHLs into the environment produced by the test organisms. For this purpose, 200 μL of temperate LB agar was added into wells of polystyrene microplates (Nunc, Roskilde, Denmark) and left to solidify. *A. tumefaciens* A136 and *C. violaceum* CV026 strains were grown in LB agar for 18 h at 30°C and suspensions from two to three colonies were made in phosphate-buffered saline (PBS) to obtain an OD_{600} of 0.05 and the wells of the microplate were inoculated with 25 μL of either *C. violaceum* CV026 or *A. tumefaciens* A136 suspensions. The plates were air dried for 30 min and 20 μL of bacterial suspensions prepared in PBS were pipetted into each well. The plates were covered and incubated at 37°C for 48 h. Positive results in each assay were determined by the bluish–green pigmentation as judged by the unaided eye. Assays were performed in triplicate in three different occasions.

PCR analysis of the QS genes

Chromosomal DNA was extracted using a commercial kit (High Pure PCR Template Kit; Roche Diagnostics, Basel, Switzerland) and oligonucleotide primers designed for *lasI*, *lasR*, *rhlI* and *rhlR* genes were used (Fermentas, Glen Burnie, MD, USA). PCR was performed in 50 μL of reaction mixture containing 30 ng of chromosomal DNA, 25 μL master mix (Fermentas), 100 pmol of forward and reverse primers (1.5 μL each), and H_2O. The PCR steps included: initial denaturation at 94°C for 7 min, followed by 34 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 2 min, and final extension at 72°C for 10 min. DNA fragments were detected on 1% agarose gels with ethidium bromide staining [9].

Antimicrobial susceptibility testing

The disk diffusion method of the CLSI was used to determine the antimicrobial susceptibility of clinical *P. aeruginosa* strains (n = 100). The *P. aeruginosa* strain ATCC 27853 was used as the internal control [20].

Statistical analysis

The correlation between the production of virulence factors and antimicrobial susceptibility was evaluated using SPSS software (SAS Institute, Cary, NC, USA) with Fisher's exact test and $p < 0.05$ was considered statistically significant.

Results and Discussion

QS-dependent virulence factors (elastase, alkaline protease, pyocyanin and biofilm) were assayed in the clinical respiratory isolates of *P. aeruginosa* (n = 100) and the control strains. Approximately 75% of the isolates were found to exhibit the QS-dependent phenotypes (Table 1). Sixteen isolates displaying impaired QS-dependent phenotypes were further analysed with a micro-AHL cross-feeding bioassay for the production of the autoinducers $3OC_{12}$-HSL and C_4-HSL, and with PCR for the detection of QS genes (*lasI*, *lasR*, *rhlI* and *rhlR*). As shown in Table 2, the biofilm forming ability differed greatly among those isolates, as previously demonstrated by Dénervaud *et al.* [10] who failed to detect any difference in biofilm formation capacity between autoinducer-deficient and -proficient strains. In the present study, both autoinducers were present in five isolates (strains 4, 8, 45, 47 and 100), whereas the remaining eleven isolates were defective either in one or in both of the autoinducers and considered QS-deficient. In five of the QS-deficient isolates that were negative for both autoinducers (strains 31, 70 and 92) or for one autoinducer (strains 81 and 84), all QS genes were present. Similar to our findings, Schaber *et al.* [9] have also identified a *P. aeruginosa* wound isolate with no elastase and pyocyanin activity, which was negative for both autoinducers but contained all QS genes. In six of our QS-deficient isolates (strains 21, 27, 39, 60, 83 and 90), one or more of the QS genes, and in three of them (strains 39, 60 and 83),

TABLE 1. Results of phenotypic tests for the investigation of virulence factor production

Virulence factor	Control strains			Clinical isolates	
	PAO1[a]	PAO-JP2, -JP3[a]	Cut-off[b]	Mean[c]	Positive (%)
Elastase (A_{495})	2.557	0.195	0.219	1.162 ± 1.045	76
Alkaline protease (A_{590})	2.671	0.217	0.241	1.473 ± 1.081	73
Pyocyanin (A_{520})	0.185	0.035	0.040	0.118 ± 0.033	75
Biofilm (A_{590})	0.124	0.065	0.076	0.119 ± 0.094	86

[a]Values are the means of tests performed in triplicate in three different occasions with an SD <10%.
[b]Cut-off values for the evaluation of virulence factor production were determined using the results of negative control strains PAO-JP2 and -JP3, and defined as mean absorbance value + 2 SD.
[c]Values are the mean ± SD of tests performed in triplicate in three different occasions for the clinical respiratory isolates of *Pseudomonas aeruginosa* (n = 100).

TABLE 2. Investigation of autoinducer production and quorum sensing (QS) genes in clinical respiratory isolates of *Pseudomonas aeruginosa* showing impaired phenotypic activity in QS-dependent factors (n = 16)

Strain	Autoinducer		QS Genes				Virulence factors[a]			
	$3OC_{12}$-HSL	C_4-HSL	*lasI*	*lasR*	*rhlI*	*rhlR*	Elastase	Alkaline protease	Pyocyanin	Biofilm
PAO1	+	+	+	+	+	+	100	100	100	100
PA-04	+	+	+	+	+	+	0	19	12	56
PA-08	+	+	+	+	+	+	27	44	16	64
PA-45	+	+	+	+	+	+	23	5	3	6
PA-47	+	+	+	+	+	+	6	34	12	1
PA-100	+	+	+	+	+	+	6	4	2	56
PA-31	–	–	+	+	+	+	3	5	4	24
PA-70	–	–	+	+	+	+	4	1	1	9
PA-81	+	–	+	+	+	+	1	1	1	9
PA-84	–	+	+	+	+	+	7	0	1	67
PA-92	–	–	+	+	+	+	5	2	23	1
PA-21	–	–	+	+	–	–	4	3	31	30
PA-27	–	–	+	+	–	–	0	0	21	66
PA-39	–	–	–	–	–	–	1	6	1	31
PA-60	–	–	–	–	–	–	1	3	3	6
PA-83	–	–	–	–	–	–	0	2	1	0
PA-90	+	–	+	+	+	–	3	0	2	10

[a]Values represent the percentages of the activity of PAO1, the positive control strain whose activity is considered as 100%.

all QS genes failed to amplify with the primers we used. The lack of PCR amplification does not by itself indicate the absence of QS genes; the target sequences might have differed substantially from the primer sequences that we used or the target genes may have been mutated or absent. Conversely, the presence of a PCR product does not exclude the possibility that the QS genes may have had inactivating mutations and this might explain the impaired QS-dependent phenotypes observed in the ten isolates in which all four QS genes were positive with PCR.

Although the production of both autoinducers and all QS genes were positive in five of the isolates (strains 4, 8, 45, 47 and 100), they failed to exhibit the QS-dependent phenotypes. Despite these results obtained *in vitro*, these strains could be more virulent at the site of infection *in vivo*. Another explanation could be that the regulation of QS systems is not limited only to *lasI*/*R* and *rhlI*/*R*, but many other global regulators are interconnected with the QS circuitry. The activation of QS requires a critical cell density, although a quorum of bacteria is not sufficient by itself to trigger the QS-regulated genes. A comprehensive review on the regulation of QS in *P. aeruginosa* is available elsewhere (21).

Respiratory isolates of *P. aeruginosa* defective in autoinducer production have also been observed in some other studies [9–12]. Among 442 *P. aeruginosa* isolates obtained in one study from 13 mechanically ventilated patients, nine different genotypes were identified; three genotypes were defective in the production of both AHLs and harboured mutations in *lasR* (three stains) and *rhlR* (two strains) [10]. In another study of CF patients, only 61% of the *P. aeruginosa* isolates were found to produce both autoinducers, whereas 75% of the isolates produced $3OC_{12}$-HSL only and 69% produced C_4-HSL only [11]. Among *P. aeruginosa* isolates obtained from critically ill patients with ventilator associated pneumonia, one-third of the isolates produced lower amounts of autoinducers [12]. Schaber *et al.* [9] also identified three *P. aeruginosa* respiratory isolates lacking the AHLs and having

mutations in QS-genes. Recent studies indicate that *lasR* is the most affected gene in CF patients that accounts for the ability of *P. aeruginosa* to adapt to the respiratory environment as the chronic disease develops [22].

Among our *P. aeruginosa* respiratory isolates, 15 were obtained from CF patients and only two (strains 39 and 90) showed impaired phenotypic activity with accompanying lack of autoinducer production and QS gene mutations. Heurlier *et al.* [23] suggested that the maintenance of a functional QS has fitness penalty in situations where such functions are not required. On the other hand, a *lasR* negative mutant could also be fitter than the wild-type in certain circumstances, such as in mixed *P. aeruginosa* infections where a QS-competent strain might provide the necessary virulence factors for the co-colonizing QS-negative strain. Köhler *et al.* [24] support this approach, suggesting that rapid adaptation is the massive fitness advantage of the *lasR* mutants, gained by exploiting the cooperating wild-type population without contributing to the public good.

A striking observation in the present study was the correlation between deficiency in virulence factor production and decreased susceptibility to antimicrobials. The isolates that were negative for virulence factor production were generally less susceptible to antimicrobials and statistically significant correlations were observed for some agents. This included a lack of elastase production and resistance to piperacillin and ceftazidime, failure of alkaline protease production and resistance to tobramycin, piperacillin, piperacillin-tazobactam, cefepime, imipenem and ciprofloxacin, and failure of pyocyanin production and resistance to amikacin, tobramycin, ceftazidime, ciprofloxacin and ofloxacin (Table 3). Considering the fact that biofilm production is partly controlled by QS and that the standard disk diffusion method does not reflect the susceptibilities of bacteria within a biofilm, the relationship between biofilm production and antimicrobial susceptibility was not added to the Table 3.

QS and antimicrobial susceptibility are closely related in *P. aeruginosa*. Efflux pump systems, which actively expel antimicrobials out the cell, contribute greatly to the intrinsic and acquired resistance of the bacterium. *P. aeruginosa* cells are freely permeable to C_4-HSL but $3OC_{12}$-HSL requires active efflux. The MexAB-OprM efflux system has shown to be involved in active efflux of $3OC_{12}$-HSL [25]. Evans *et al.* [26] demonstrated that strains overexpressing the MexAB-OprM efflux secrete less $3OC_{12}$-HSL, pyocyanin, elastase and casein protease compared to wild-type. In agreement with our findings, Köhler *et al.* [27] reported that mutants overexpressing the MexEF-OprN efflux system produced lower levels of QS-dependent virulence factors, pyocyanin, elastase and rhamnolipids.

In conclusion, we suggest that QS-deficient *P. aeruginosa* strains may produce respiratory infections and tend to be less susceptible to antimicrobials. This could be a drawback for the clinical use of QS-inhibitory drugs that appear superior to conventional antimicrobials by not exerting any selective pressure on resistant strains.

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TABLE 3. The correlation between the production of quorum sensing-dependent virulence factors and antimicrobial susceptibility in clinical *Pseudomonas aeruginosa* isolates (*n* = 100)

	Elastase			Alkaline protease			Pyocyanin		
Rates of resistance to antimicrobials (%)	Positive (*n* = 76)	Negative (*n* = 24)	p-value	Positive (*n* = 73)	Negative (*n* = 27)	p-value	Positive (*n* = 75)	Negative (*n* = 25)	p-value
Amikacin	29.2	30.3	0.999	27.4	37.0	0.461	22.7	52.0	0.011
Gentamicin	39.5	41.7	0.999	34.2	55.6	0.067	36.0	52.0	0.167
Tobramycin	30.3	33.3	0.803	21.9	55.6	0.003	24.0	52.0	0.013
Piperacillin	25.0	50.0	0.041	20.5	59.3	0.001	28.0	40.0	0.320
Piperacillin/tazobactam	19.7	20.8	0.999	11.0	44.4	0.001	16.0	32.0	0.093
Ceftazidime	17.1	37.5	0.048	16.4	37.0	0.054	16.0	40.0	0.023
Cefepime	26.3	33.3	0.603	19.2	51.9	0.002	24.0	40.0	0.132
Imipenem	31.6	41.7	0.459	26.0	55.6	0.009	32.0	40.0	0.475
Meropenem	21.1	29.2	0.415	17.8	37.0	0.060	20.0	32.0	0.273
Ciprofloxacin	28.9	41.7	0.316	23.3	55.6	0.003	22.7	60.0	0.001
Ofloxacin	44.7	45.8	0.999	41.1	55.6	0.258	34.7	76.0	0.001

Transparency Declaration

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