Antibiotic Resistance Profiles and Quorum Sensing-Dependent Virulence Factors in Clinical Isolates of *Pseudomonas Aeruginosa*

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Abstract *Pseudomonas aeruginosa* produces multiple virulence factors that have been associated with quorum sensing. The aim of this study was to evaluate the prevalence of drug resistant profiles and quorum sensing related virulence factors. *Pseudomonas aeruginosa* were collected from different patients hospitalized in China, the isolates were tested for their susceptibility to different common antimicrobial drugs and detected QS-related virulence factors. We identified 170 isolates displaying impaired phenotypic activity, approximately 80 % of the isolates were found to exhibit the QS-dependent phenotypes, among them, 12 isolates were defective in AHLs production, and therefore considered QS-deficient strains. Resistance was most often observed to Cefazolin (81.2 %), followed by trimethoprim–sulfamethoxazole (73.5 %), ceftriaxone (62.4 %) and Cefotaxime, Levofloxacin, Ciprofloxacin (58.8 %), and to a lesser extent Meropenem (20.0 %), Cefepime (18.8 %), and Cefoperazone/sulbactam (2.4 %). The QS-deficient isolates that were negative for virulence factor production were generally less susceptible to the antimicrobials. The results showed a high incidences of antibiotic resistance and virulence properties in *P. aeruginosa*, and indicate that the

clinical use of QS-inhibitory drugs that appear superior to conventional antimicrobials by not exerting any selective pressure on resistant strains.

Keywords *Pseudomonas aeruginosa* · Drug resistance · Quorum sensing · Virulence factors

Introduction

Pseudomonas aeruginosa is a gram negative, asporogenous, obligate aerobic, motile and oxidase positive bacilli, usually found in the intestinal tract, water, soil and sewage [1, 2]. It is an opportunistic pathogen that is a common cause of hospital-acquired infections, particularly infecting patients with predisposing factors, such as burn victim, immunocompromised hosts, or those with metabolic disorders. 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. The pathogenesis of *P. aeruginosa* is multifactorial and depends on numerous virulence factors, including the secreted factors such as elastase, alkaline protease, exotoxin, pyocyanin, pyoverdine, hydrogen cyanide, rhamnolipid and cell-associated factors, such as lipopolysaccharide, flagella and pili [3]. Another factor contributing to pathogenesis of *P. aeruginosa* is its tendency to form organized communities, known as biofilms. Moreover, *P. aeruginosa* strains exhibit high rates of resistance to antibiotics and are frequently multidrug resistant [4, 5]. This high incidence of resistance causes several therapeutic complications and is associated with treatment failure and death. Thus, surveillance cultures of antibiotic resistant *P. aeruginosa* strains

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from the rectum, stomach, oropharynx, trachea, or skin are usually done on admission and during hospitalization in intensive care units (ICUs).

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) [6]. QS is a cell-density-dependent mechanism through which bacteria coordinate different activities including bioluminescence, plasmid conjugation and the production of different virulence factors [7]. *P. aeruginosa* possesses at least two well-defined, interrelated QS systems, *las* and *rhl*, that control the production of different virulence factors, including elastases (LasB and LasA), alkaline protease, hydrogen cyanide, exotoxin A, pyocyanin, lectins, rhamnolipids and superoxide dismutase. Each QS system consists of two components, the autoinducer synthases (LasI and RhlI, respectively) and their cognate transcriptional regulators (LasR and RhlR, respectively). LasI is the synthase for the autoinducer *N*-(3-oxododecanoyl) homoserine lactone (3OC12-HSL), while RhlI synthesizes the autoinducer *N*-butyryl homoserine lactone (C4-HSL). At high cell density, 3OC12-HSL and C4-HSL reach critical levels and activate their regulators, which in turn enhance the transcription of different virulence genes [8–10].

In the present study, we aimed to assess the activity of QS-dependent virulence factors in clinically pathogenic isolates of *P. aeruginosa* and their relationship with antimicrobial susceptibility, another important virulence factor of *P. aeruginosa*.

Materials and Methods

Isolation and Identification of *P. aeruginosa*

From January 2011 to May 2012, 170 *P. aeruginosa* isolates were collected from different patients hospitalized at Lishui People's Hospital, Zhejiang province and the Affiliated Hospital of North Sichuan Medical College, Sichuan province. *P. aeruginosa* was identified biochemically from routinely obtained specimens by means of the Vitek ATB Expression System, version 2.7.8 (BioMérieux Deutschland GmbH, Nürtingen, Germany), which uses 32 biochemical reactions. Bacterial isolates were stored as suspensions in a 10 % (wt/vol) sterilized milk solution containing 12.5 % (vol/vol) glycerol at -70°C until tests were performed.

Antibiotic Resistance Assays

The antimicrobial sensitivity test of each isolate was carried out by the Kirby-Bauser disc diffusion method [11] as per

recommendation of National Committee for Clinical Laboratory Standards [12]. The following antimicrobials were tested: Cefazolin (CZO), ceftriaxone (CRO), cefotaxime (CTX), ceftazidime (CAZ), cefepime (CEP), Cefepime (FEP), Cefuroxime sodium (CXM), Cefoperazone/sulbactam (SCF), Piperacillin—tazobactam (TZP), Imipenem (IPM), Meropenem (MEM), Azithromycin (AZM), Amikacin (AMK), Tobramycin (TOB), Gentamicin (GEM), Levofloxacin (LEV), Ciprofloxacin (CIP), Trimethoprim/sulphamethoxazole (SXT). Cartridges of antimicrobial containing discs were obtained from Shanghai Yihua Medical Science & Technology Co., Ltd. (Shanghai, China), stored between 4 and -20°C , and allowed to come to room temperature prior to use. Isolates were subcultured from the bank onto Miller's LB agar and incubated for 18–24 h before being transferred to 5 ml sterile 0.9 % saline to match the '0.5' MacFarland standard (Shanghai Ruiqi Biological Technology Co. Ltd, Shanghai, China). A sterile cotton-tipped swab was used to streak air-dried Mueller–Hinton II plates within 15 min of adjustment of turbidity. Subsequently, antimicrobial discs were added and plates were incubated aerobically at $35 \pm 2^{\circ}\text{C}$ for 16–18 h. The diameter of the zones of inhibition surrounding the antimicrobial discs was measured to the nearest mm. Isolates were deemed resistant only when the zone of inhibition was less than or equal to the resistance breakpoint recommended by the NCCLS guidelines. Quality control was performed as recommended using *P. aeruginosa* strain ATCC 27853.

Virulence Markers Assays

Cross-feeding Bioassay for AHLs

Cross-feeding bioassay method was used to detect AHLs production. Detection of AHLs was achieved using either the *Chromobacterium violaceum* strain CV026 or the *Agrobacterium tumefaciens* strain A136 were used as biosensors [13]. *C. violaceum* CV026 (a mini-Tn5 mutant) was used as an indicator strain for the detection of C4-HSL. *C. violaceum* CV026 responds AHLs by inducing the synthesis of the purple pigment violacein. *A. tumefaciens* strain A136, carrying the plasmid (pCF218)(pCF372), was used to detect 3O C12-HSL. *A. tumefaciens* A136 produces a blue color in the presence of X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) in response to the AHLs with *N*-acyl chain length from six to 12 carbons. These strains were grown in or on LB (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl) solidified with 1.2 % agar when required and supplemented with appropriate antibiotics (*A. tumefaciens* A136 spectinomycin 50 $\mu\text{g/ml}$ and tetracycline 4.5 $\mu\text{g/ml}$; *C. violaceum* Kanamycin 20 $\mu\text{g/ml}$). *P. aeruginosa* PAO1 as positive control strain for AHLs production.

Elastase Assay

Elastase activity was measured using the elastin Congo red (ECR, Sigma) assay [14]. Cells were grown in LB broth at 37 °C for 14 h. A 100 µl aliquot of bacterial supernatant was added to 900 µl of ECR buffer (100 mM Tris, 1 mM CaCl₂, pH 7.5) containing 20 mg of ECR and incubated with shaking at 37 °C for 3 h. 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

LB agar plates containing 0.2 g cetyltrimethylammonium-bromide (CTAB) and 5 mg methylene blue 1–1 were inoculated with 2 µl 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 [15].

Protease Activity

Protease production and proteolytic activity was detected on 1.2 % agar plates supplemented with 10 % (v/v) sterile skimmed milk (105 °C for 30 min). The cultures were streaked on the skim milk agar plates and incubated at 27 °C for 24–36 h. Proteolytic strains caused a clearing zone around the colonies.

Assay for Pyocyanin Production

Pyocyanin was extracted from culture supernatants and measured by the method of Essar et al. [16]. A 3 ml volume of chloroform was added to 5 ml of culture supernatant and mixed. The chloroform layer was transferred to a fresh tube and mixed with 1 ml of 0.2 M HCl. After centrifugation, the top layer (0.2 M HCl) was removed. The amount of pyocyanin within the extracted layer was determined by measuring A520.

Biofilm Assay

A quantitative biofilm formation experiment was performed in a microtiter plate as described previously [17], with minor modification. Briefly, bacteria were grown on LB agar, and several colonies were gently re-suspended in LB (with or without the appropriate antibiotic); 100 µl aliquots were placed in a microtiter plate (polystyrene) and incubated 48 h at 28 °C without shaking. After the bacterial cultures were poured out, the plate was washed extensively with water, fixed with 2.5 % glutaraldehyde, washed once with water, and stained with a 0.4 % crystal

violet solution. After solubilization of the crystal violet with ethanol-acetone (80:20, vol/vol) the absorbance at 590 nm was determined using a microplate reader (Bio-Rad, Hercules, Calif.).

Results and Discussion

A collection of 170 non-duplicate *P. aeruginosa* isolates were obtained from wounds, sputum, urine, high vaginal swab (HVS), ear, eye and respiratory tract swabs, and aspirates of peritoneum and ulcers submitted by hospitalized patients. *P. aeruginosa* was identified biochemically by means of the Vitek ATB Expression System, version 2.7.8 (BioMérieux Deutschland GmbH, Nürtingen, Germany), which uses 32 biochemical reactions. Among the isolates there were 12 strains can not produce AHLs based on Cross-feeding bio-assay using either the *C. violaceum* strain CV026 or the *A. tumefaciens* strain A136 were used as biosensors.

Antimicrobial Susceptibility of *P. aeruginosa* Isolates

One of the virulence attributes of *P. aeruginosa* is its resistance to different antibiotics. This resistance is provided by several multi-drug resistance (MDR) pumps. It has been shown that the resistance of a specific *P. aeruginosa* strain to antibiotics varies depending on the number of MDR pumps expressed. We examined the resistance of the all 170 isolates to antimicrobial agents used in the hospital in accordance with NCCLS methods. Susceptibility testing of the 170 *P. aeruginosa* isolates showed that all the isolates were resistant to at least one of the antibiotics. Resistance was most often observed to Cefazolin (81.2 %), followed by trimethoprim—sulfamethoxazole (73.5 %), ceftriaxone (62.4 %) and Cefotaxime, Levofloxacin, Ciprofloxacin (58.8 %), and to a lesser extent Meropenem (20.0 %), Cefepime (18.8 %), and Cefoperazone/sulbactam (2.4 %) (Table 1).

Prevalence of QS-dependent Virulence Factors of *P. aeruginosa* Isolates

P. aeruginosa causes many different types of infections in humans like cystic fibrosis, burn wound, respiratory tract infections, microbial keratitis and urinary tract infection, is multi-factorial in its virulence. Depending on the infected tissue, some virulence factors play more important roles in the pathogenesis of *P. aeruginosa* infections than others. The contribution of individual factors to *P. aeruginosa* virulence in natural infections has been examined by several clinical studies. In this study, QS-dependent virulence factors (elastase, protease, pyocyanin, rhamnolipids and biofilm formation) were assayed in the clinical isolates of

Table 1 Antimicrobial susceptibility testing results of 170 isolates of *P. aeruginosa* collected from two hospitals

Antibiotic	Sensitive no. (%)	Intermediate no. (%)	Resistant no. (%)
Cefazolin (30 µg)	32/170 (18.8 %)	0	138 (81.2 %)
Ceftriaxone (30 µg)	30 (17.7 %)	34 (20 %)	106 (62.4 %)
Cefotaxime (30 µg)	31 (18.2 %)	39 (23 %)	100 (58.8 %)
Ceftazidime (30 µg)	84 (49.4 %)	15 (8.8 %)	71 (41.8 %)
Cefepime (30 µg)	90 (53 %)	32 (18.8 %)	48 (28.2 %)
Cefepime (30 µg)	120 (70.6 %)	18 (10.6 %)	32 (18.8 %)
Cefuroxime sodium (30 µg)	36 (21.2 %)	48 (28.2 %)	86 (50.6 %)
Cefoperazone/sulbactam (75/75)	130 (76.5 %)	36 (21.2 %)	4 (2.4 %)
Piperacillin/tazobactam (100/10)	128 (75.3 %)	0	42 (24.7 %)
Imipenem (10 µg)	119 (70 %)	7 (4.1 %)	44 (25.9 %)
Meropenem (10 µg)	128 (75.3 %)	8 (4.7 %)	34 (20 %)
Azithromycin (35 µg)	80 (47.1 %)	33 (19.4 %)	57 (33.5 %)
Amikacin (30 µg)	105 (61.8 %)	16 (9.4 %)	49 (28.8 %)
Tobramycin (10 µg)	106 (62.4 %)	6 (3.5 %)	58 (34.1 %)
Gentamicin (10 µg)	64 (37.7 %)	7 (4.1 %)	99 (58.2 %)
Levofloxacin 5 µg	64 (37.7 %)	6 (3.5 %)	100 (58.8 %)
Ciprofloxacin (5 µg)	66 (38.8 %)	4 (2.4 %)	100 (58.8 %)
Trimethoprim/sulphamethoxazole (23.75/1.25 µg)	33 (19.4 %)	12 (7.1 %)	125 (73.5 %)

P. aeruginosa ($n = 170$) and the control strains. Approximately 80 % of the isolates were found to exhibit the QS-dependent phenotypes (Table 2). Clinical isolates deficient in the production of virulence factors regulated by QS, such as elastase and exotoxin A, have been described previously [18, 19]. However, only few data exist concerning QS-deficient clinical isolates of *P. aeruginosa* [20, 21]. In this study, we isolated 12 strains which can not produce AHLs based on cross-feeding bioassay method. These isolates lost most of their known virulence factors, but they still cause blood, urinary tract and wound infections in humans.

One of the virulence attributes of *P. aeruginosa* is its resistance to different antibiotics. Bacteria imbedded in biofilms are more tolerant to several antibiotics than their planktonic bacteria counterparts [22, 23]. In this study, we found 89 % isolates can form biofilm leads to more tolerant to antibiotics, and 12 isolates which can not produce AHLs also can form biofilm were less susceptible to antimicrobials. This finding is according with Karatuna and Yagci

reports [20]. The QS-deficient biofilm was almost entirely eradicated, in contrast with the wild-type biofilm, in which only cells in the top layer were killed. Karatuna and Yagci results showed that QS-deficient strains were tend to be less susceptible to antimicrobials by comparing wild-type [24]. Interestingly, sub-inhibitory concentrations of ceftazidime and tobramycin have been found to reduce the amounts of QS signal molecules in *P. aeruginosa* [25] and thereby indirectly affect QS. The QS-regulated genes involved in the increased tolerance of antibiotics and detergents remains to be elucidated. So from our results and others, QS play an important role in the pathogenicity in *P. aeruginosa*. Inhibit the QS activity can decreased the secretion of virulent factors, and it will be a promising pathway to control bacterial infection [26–29]. We have selected some traditional Chinese herb medicines which can inhibit the *P. aeruginosa* quorum sensing and found that the secretion of virulent factors and biofilm formation was decreased (It will published in another article).

In conclusion, the study of the epidemiology of antibiotic resistance and QS-dependent virulence factors of *P. aeruginosa* strains isolated during January 2011 to May 2012 in two Hospital in China showed a high incidences of antibiotic resistance and virulence properties independently of their origin. We also found that QS-deficient *P. aeruginosa* strains 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.

Table 2 Results of phenotypic tests for the investigation of virulence factor production

Virulence factor	PAO1	Clinical isolates (%)
Elastase (A495)	0.247	82
Pyocyanin (A520)	0.165	78
Biofilm (A590)	0.545	89
Protease activity	+	81
Rhamnolipids	+	79

Conflict of interest We declare that we have no conflict of interest.

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