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Original Article

Association between the *Pseudomonas aeruginosa* type III secretion system, antibiotic resistance and clinical features

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Abstract: *Pseudomonas aeruginosa* (*P. aeruginosa*) is an important agent of human infections. The type III secretion system (TTSS) has recently been recognized as an important virulence factor that plays a major role in the pathogenesis of serious *P. aeruginosa* infections. The virulence genes, drug resistance, and drug-resistant gene mutations were determined in 152 *P. aeruginosa* samples isolated between January and June 2015. The TTSS-positive rate was 71.1% (108/152). Sixty-six of the 100 (66%) multidrug-resistant (MDR) strains and 42 of the 52 (80.77%) non-MDR strains were TTSS positive. The TTSS-positive rate of non-MDR strains was higher than that of the MDR strains, but there was no statistically significance ($P > 0.05$). The drug resistance rates of the TTSS-negative strains for all 13 drugs tested were higher than those of the TTSS-positive strains, but there were no significant differences observed between these groups ($P > 0.05$). The mutation rates of the β-lactam drug-hydrolyzing enzymes IMP-1, IMP-2, VIM-1, and VIM-2 were 19.7%, 14.5%, 13.2% and 13.2%, respectively. The mutation rates of drug resistance genes of the TTSS-negative strains were higher than those of the TTSS-positive strains. The data demonstrated that there was no relationship between the TTSS virulence factors and drug resistance.

Keywords: *Pseudomonas aeruginosa*, type III secretion system, virulence, multidrug-resistance, gene mutations

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an important causative agent of human infections, especially in a host with a compromised defense system [1]. Severe infections, such as bloodstream infections and ventilator-associated pneumonia, due to multidrug-resistant (MDR) *P. aeruginosa* infection, result in greater morbidity and mortality, longer hospitalizations, and higher costs than infections caused by drug-susceptible bacteria [2, 3].

The virulence factors of the bacterium could be chemical or proteinaceous, and either cell-associated or secreted. Proteinaceous virulence factors were often secreted through one of the five protein secretion systems that have been described in *P. aeruginosa*: types I, II, III, and V, and the recently discovered type VI [4]. In particular, the type III secretion system (TTSS) has been associated with high virulence [5]. Infection with a type III effector-secreting isolate has been shown to be correlated with severe disease [6], and the TTSS in low respiratory and systemic infections was associated with an increased mortality rate. Once host cell was contacted, the TTSS allowed bacteria to directly inject toxins into the host cell via a translocation complex, which could further induced cell necrosis and modulation of the actin cytoskeleton, allowing the bacteria to invade the eukaryotic cells and avoid phagocytosis [5-8], whereupon they subverted the host cell defense and signaling systems. The four well-known TTSS effector molecules were exoenzymes (Exo) S, U, T, and Y [9].

ExoS was a major cytotoxin involved in the colonization, invasion, and dissemination stages of infection [10]. ExoU was a potent cytotoxin with phospholipase activity and capable of killing a variety of eukaryotic cells *in vitro*. Additionally, ExoU had a greater virulence than other TTSS effectors of the bacteria [8].

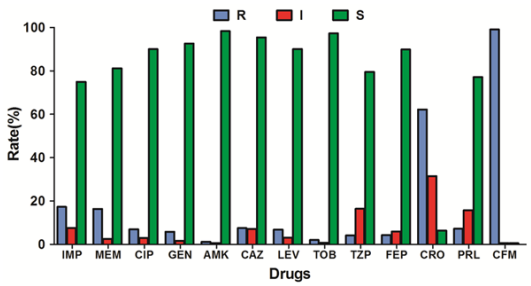

Figure 1. Prevalence of antimicrobial resistance phenotypes of *P. aeruginosa* isolated from Zhongshan in 2015. A total of 495 P. aeruginosa strains isolated from patients in 2015 were resistant to a wide range of antimicrobial drugs. The resistance rates of the isolates against the other drugs were all lower than 20% except for ceftriaxone and cefuroxime. LVX: levofloxacin; MEM: meropenem; AMK: amikacin; PIP: piperacillin; IMP: imipenem; GEN: gentamicin; CIP: ciprofloxacin; TOB: tobramycin; TZP: piperacillin/tazobactam; FEP: cefepime; CAZ: ceftazidime; CRO: ceftriaxone; CFM: cefuroxime.

As the presence of TTSS-encoding genes in clinical isolates of *P. aeruginosa* was still unknown, the distribution of these genes in different populations needed to be studied. Moreover, to the best of our knowledge, there was no previous report investigating the prevalence of *P. aeruginosa* virulence genes in strains from Zhongshan, China. Therefore, the present study aimed to evaluate the frequencies of the *exoT*, *exoY*, *exoS*, and *exoU* genes among different clinical isolates of *P. aeruginosa* in this region of China. We also sought to determine whether there is any correlation between the presence of these genes and the antibiotic resistance phenotype and genotype profiles of the isolates.

Materials and methods

P. aeruginosa specimens

This study included 152 *P. aeruginosa* samples isolated between January and June 2015 from 154 patients from different departments of Zhongshan Hospital (Sun Yat-Sen University, Guangdong, China). The clinical and drug resistance data were sorted using WHONET 5.6 software.

Strains

Identification of *P. aeruginosa* and the drug resistance phenotype was performed using the VITEK 2 Compact system (bioMerieux, France). The drugs analyzed in this article were levofloxacin, meropenem, amikacin, piperacillin, imipenem, gentamicin, ciprofloxacin, tobramycin, piperacillin/tazobactam, cefepime, ceftazidime, ceftriaxone, and cefuroxime. Results were interpreted according to Clinical and Laboratory Standards Institute criteria.

Detection of drug resistance genes and virulence genes encoding TTSS effectors

The cultures were inactivated at 100°C for 5 min, and DNA was extracted from the cells by the cetyltrimethylammonium bromide-phenol method [11]. The primers used to detect the genes encoding the metallo-β-lactamases IMP-1, IMP-2, VIM-1, and VIM-2 were as described before [12]. The virulence genes *exoY*, *exoS*, *exoT* and *exoU* were amplified by the polymerase chain reaction (PCR) method [13].

PCR assay

Each PCR assay was conducted in a total volume of 20 μL containing 10 μL of 2 × Premix Taq Version 2.0 (loading dye mix) (TaKaRa, China), 0.4 μL of each primer, 1 μL of DNA, and 8.2 μL of double-distilled water. The PCR conditions were as follows: an initial denaturation step at 94°C for 10 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. Then the PCR assay was conducted in a C1000 Thermal Cycler (Bio-Rad, USA). The products were analyzed by electrophoresis on a 1.5% agarose gel, stained with 5% (w/v) ethidium bromide (EB) solution and visualized under ultraviolet light. The copy number at each band was calculated using ImageLab Software (Bio-Rad).

Statistical analysis

Statistical analyses of the associations were performed using SPSS 21.0. The chi-squared test was used to determine the differences between the two groups. $P < 0.05$ was considered statistically significant.

Results

Drug resistance of P. aeruginosa

A total of 495 *P. aeruginosa* strains were isolated from patients in 2015. These strains were

Table 1. Different distribution of exo genes in isolates with different origins

Virulence gene	Sputum (*n* = 50)	Urine (*n* = 30)	Wound (*n* = 8)	Blood (*n* = 32)	Burn (*n* = 20)	Pus (*n* = 12)	Total (*n* = 152)
exoS	40 (80)	20 (66.7)	2 (25)	4 (31.25)	10 (50)	6 (50)	82 (53.95)
exoY	35 (70)	21 (70)	3 (37.5)	19 (59.375)	7 (35)	5 (41.67)	90 (59.21)
exoT	4 (8)	1 (6.7)	2 (25)	2 (6.25)	2 (10)	1 (8.33)	12 (7.89)
exoU	4 (8)	3 (10)	1 (12.5)	4 (12.5)	2 (10)	2 (16.67)	16 (10.53)

Table 2. Distribution of the virulence genes of TTSS positive strains (*n* = 108)

Types	NO. of strains	Virulence genes				%
		exoU	*exoS*	*exoT*	*exoY*	
1	64	-	+	-	+	59.26
2	10	+	-	-	+	9.26
3	10	-	+	-	-	9.26
4	8	-	-	-	+	7.41
5	6	-	-	+	-	5.56
6	4	-	+	+	+	3.70
7	2	+	-	+	+	1.85
8	2	+	+	-	-	1.85
9	2	+	+	-	+	1.85
Sum	108	16	82	12	90	100

resistant to a wide range of antimicrobial drugs. Except for ceftriaxone and cefuroxime, the resistance rates of the isolates against the other drugs were all lower than 20% (**Figure 1**). The drug resistance rate in this region of China has in fact decreased year by year. Carbapenems are the last resort for treatment [14], and a systematic review and meta-analysis had shown that carbapenem use and medical devices were the leading risk factors for carbapenem-resistant *P. aeruginosa* [3]. The resistance rates for carbapenems, fluoroquinolones, and aminoglycosides in 2015 were significantly lower than those in 2010 (17.6%, 6.1%, 2.1% vs. 25%, 30%, 36.8%, respectively).

Distribution of the TTSS genes

The distribution of virulence genes among different clinical samples was shown in **Table 1**. Of the 152 patient samples, 37 (24.0%) were from surgery intensive care units, 102 (67.1%) were from sterile body fluids, and 50 (32.5%) were from blood. The four virulence genes were detected among all the isolates. However, the relative frequency of *exoS* was higher in the sputum (80%) and urine (66.7%) samples than in the other clinical samples. Although the relative frequencies of the virulence genes were different among clinical specimens, statistical analysis showed that there were no differences in the presence of individual genes and sources of isolation. The type III secretion toxin-encoding gene patterns were shown in **Table 2**. The TTSS-positive rate was 71.1% (108/152). With regard to gene distribution, 59.2% (90/152) of the samples carried *exoY*, 10.5% (16/152) carried *exoU*, 53.9% (82/152) carried *exoS*, and 7.9% (12/152) carried *exoT*. Coexistence of *exoS*, *exoY*, and *exoU* was observed in 4% of the isolates. There were nine types in the 108 TTSS-positive strains. Seventy-six strains carried two virulence genes, where 9.21% (14/152) of the isolates carried both *exoY* and *exoU*, 46.05% (70/152) carried both *exoS* and *exoY*, 2.63% (4/152) carried both *exoS* and *exoT*, and 2.63% (4/152) carried both *exoS* and *exoU*. Eight strains carried three virulence genes at the same time (4 of *exoU-/exoS+/exoT+/exoY+*, 2 of *exoU+/exoS+/exoT-/exoY+*, and 2 of *exoU+/exoS-/exoT+/exoY+*). The others strains only carried one type of the virulence gene, and there were no strains carrying all four genes.

Analysis of the virulence genes between MDR strains and non-MDR strains

In our investigation, 65.8% of the resistant isolates exhibited a MDR phenotype. Among the MDR strains, 66% (66/100) were TTSS positive, and 62.0% (62/100) carried *exoY*. Of the non-MDR strains, 80.77% (42/52) were TTSS positive, 65.4% carried *exoS*, and 65.4% had *exoY*. The TTSS-positive rate of the non-MDR strains was higher than that of the MDR strains, but there was not statistically significant ($P > 0.05$) (**Table 3**).

Analysis of the drug resistance and virulence genes

The drug resistance rate of TTSS-negative strains against all 13 drugs was higher than

Table 3. Characteristics of the virulence genes between MDR strains and non-MDR strains

Virulence genes	TTSS positive	MDR strains (*n* = 100)	Non-MDR strains (*n* = 52)	X^2	*P*
ExoS	80 (52.6)	46 (46.0)	34 (65.4)	2.578	0.108
ExoY	92 (63.2)	62 (62.0)	34 (65.4)	0.084	0.772
ExoT	12 (7.9)	4 (4.0)	8 (15.4)	1.684	0.194
ExoU	16 (10.5)	10 (10.0)	6 (11.5)	0.000	1.000

TTSS: the type III secretion system.

Table 4. Analysis of the drug-resistance rates between TTSS positive and negative strains

Drugs	TTSS Positive (*n* = 108)	TTSS Negative (*n* = 44)	X^2	*P*
IMP	64 (59.3)	34 (77.3)	0.001	0.970
MEM	33 (30.5)	18 (40.9)	1.503	0.220
CIP	30 (27.8)	16 (36.3)	1.092	0.296
LVX	31 (28.7)	16 (36.3)	0.859	0.354
GEN	31 (28.7)	16 (36.3)	0.859	0.354
AMK	31 (28.7)	16 (36.3)	0.859	0.354
TOB	31 (28.7)	16 (36.3)	0.859	0.354
ATM	34 (31.5)	12 (27.2)	0.262	0.608
CAZ	33 (30.5)	28 (63.6)	14.240	0.000
FEP	27 (25.0)	15 (34.1)	1.292	0.256
CRO	30 (27.8)	16 (36.3)	1.092	0.296
PIP	29 (26.8)	14 (31.8)	0.380	0.538
TZP	47 (43.5)	19 (43.2)	0.001	0.970

LVX: levofloxacin; MEM: meropenem; AMK: amikacin; PIP: piperacillin; IMP: imipenem; GEN: gentamicin; CIP: ciprofloxacin; TOB: tobramycin; TZP: piperacillin/tazobactam; FEP: cefepime; CAZ: ceftazidime; CRO: ceftriaxone; ATM: aztreonam.

that of the TTSS-positive strains (**Table 4**), but the difference between the two groups was not statistically significant ($P > 0.05$).

We also investigated the relationship between β-lactam drug resistance genes and virulence genes (**Table 5**). In this study, 98 isolates were unsusceptible to imipenem and/or meropenem. Among those, 50 (51.0%) isolates were metallo-β-lactamase producers. The number of isolates carrying the *bla*IMP-1 and *bla*VIM-1 genes, either alone or in combination, was 52 (53.1%) and 40 (40.8%), respectively. Eight strains (16.0%) carried more than one gene simultaneously. The mutation rates of β-lactam genes coding for IMP-1, IMP-2, VIM-1, and VIM-2 were 19.7%, 14.5%, 13.2% and 13.2%, respectively. There was no significant difference in gene mutations between the TTSS-positive and TTSS-negative strains ($P > 0.05$).

Discussion

P. aeruginosa caused a wide range of infections, including septicemia, pneumonia, endocarditis, burn wound infections and keratitis. The TTSS has recently been recognized to be one of the important virulence factors [5, 7]. It is a contact-dependent protein secretion pathway that plays a major role in the pathogenesis of serious *P. aeruginosa* infections [8]. As it was important to detect virulence genes in clinical isolates of *P. aeruginosa*, the present study sought to characterize the frequency of several *P. aeruginosa* virulence genes among different clinical isolates from patients in Zhongshan, China.

Currently, the emergence of MDR *P. aeruginosa* is becoming a global problem, resulting in the ability of this pathogen to develop resistance to almost all available antibiotics, either by selection of mutations in chromosomal genes or from horizontal gene transfer [15]. This problem was highly significant worldwide, and some studies showed a heavy use of antibiotics, especially of carbapenems and fluoroquinolones. The bacterial production of metallo-β-lactamases encoded by several genes was considered the main mechanism of resistance to these antibiotics.

A key determinant of *P. aeruginosa* was its remarkable resistance to antibiotics, and notably many of the isolates were MDR strains. In several studies, the relationship between MDR isolates and the presence of genes encoding the TTSS, especially *exoU*, has been demonstrated.

Different frequencies of cytotoxin-encoding genes, however, have been reported in different studies. We have summarized the data from different areas of the world (**Table 6**). In our study, the TTSS-positive rate was 71.1%, higher than that from urinary tract infections in pediatric patients [16]. In most of the studies, the presence of the *exoT* gene was not investigated, because previous reports had already documented the presence of the marker in nearly all *P. aeruginosa* strains from both clinical and environmental sources [7, 14, 17]. In the present study, however, the prevalence of

Table 5. Characteristics of β-lactam drug resistance genes between TTSS positive strains and TTSS negative strains

Resistance genes	Total strains	TTSS positive strains (*n* = 108)	TTSS negative strains (*n* = 44)	X^2	*P*
IMP-1	30 (19.7)	20 (18.5)	10 (22.7)	0.350	0.554
IMP-2	22 (14.5)	14 (13.0)	8 (18.2)	0.688	0.407
VIM-1	20 (13.2)	15 (13.9)	5 (11.4)	0.174	0.676
VIM-2	20 (13.2)	13 (12.0)	7 (15.9)	0.410	0.552

IMP: Imipenem; VIM: Verona integron-encoded metallo-β-lactamase.

Table 6. Characteristics of TTSS genes from different areas of the world

Area/Genes	exoY	exoT	exoU	exoS
Zhongshan, China[&]	59.2	7.9	10.5	53.9
Tianjin, China [20]			13.95	86.05
Northwest Iran [18]	55	5	52	26.3
Shiraz, Iran [2]			86	66
Guangzhou, China [13]			21.1	76.4
California, USA [5]			27	62
Bugaria [14]	85.8	100	32.4	61.9
Tehran, Iran [17]	95	100	64.5	29
USA [7]	89	100	28	71
Iran [21]				67.64
Australian [10]			17.5	97
France [26]			30.2	62.4
Shenzhen, China [19]	84.21	0	34.21	57.89

[&]The data was from our study.

the *exoT* gene was only 7.9%, similar to the result of a study conducted in Northwest Iran (5%) [18]. The *exoT* gene was not detected in strains from Shenzhen, China [19]. The *exoY* gene showed the highest prevalence (59.2%) in our study but occurred less frequently than in studies done in Bulgaria (85.8%) [14], the USA (89%) [7], and Shenzhen, China [19]; however, its frequency was similar to that in Northwest Iran [18].

Unlike other studies, we observed a lower prevalence of *exoU* (10.5%). Jabalameli and his research group detected a prevalence rate of 64.5% for *exoU* [17], but in many other areas, including the USA (27%) [5], Australia (17.5%) [10], and other areas of China (Tianjin 13.95%, Guangzhou 21.1%) [13, 20], the rates were similar to our result. This revealed the regionalism of the strains. Another study regarding the isolates from Iranian hospital infections showed that the prevalence rate could be 67.64% for *exoS* [21], whereas another research published a rate of 73.91% [22]. Interestingly, in a survey in France, the prevalence of *exoS* was markedly higher than that in other studies (94% in cystic fibrosis (CF) isolates vs. 80% in non-CF isolates) [23]. Nevertheless, these rates were all higher than that obtained this study (53.9%). Another study by Hu's research group about the TTSS in *P. aeruginosa* isolates from chronically infected older children and adults with CF showed a predominantly *exoS+/exoU-* (*exoS+*) genotype and loss of TTSS effector secretion over time [24]. The *exoU+* environmental strains were significantly less than the clinical strains to secrete ExoU. This lower rate of *exoS* and *exoU* prevalence in our study could be due to a lower clonal diversity of the isolates.

It has been suggested [21] that isolates from the clinical setting contained either the *exoS* or *exoU* gene, but not both. However, interestingly, in Northwest Iran, 48.7% and 15.3% of the isolates had *exoS+/exoU+* and *exoS-/exoU-*genotypes, respectively [18]. It was also shown that 75% of the isolates were *exoS+/exoU+* [25]. In our study, four strains (3.7%) contained two genes simultaneously. These four stains were all from the neurosurgery ward in the same period, and had the same drug resistance phenotype, and may have been a nosocomial infection.

We analyzed the relationship between the TTSS genes and drug resistance and found that the drug resistance rate of TTSS-negative strains was higher than that in the TTSS-positive strains, but the difference was not statistically significant ($P > 0.05$). The result was supported by those obtained in Guangzhou [13].

In our investigation, 65.8% of the resistant isolates exhibited a MDR phenotype, which was almost consistent with the 57.5% prevalence found in Bulgaria [26]. It has been suggested that clinical isolates carrying the *exoU* gene were significantly associated with the MDR phenotype [21, 27], but this was not coincident

with our study, since the 10% MDR resistance rate in the *exoU*+ isolates was lower than the 46% rate in the *exoS*+ isolates. We generally found no significant difference in the relationship between the TTSS status and MDR strains ($P > 0.05$).

The *bla*IMP-, *bla*VIM-, *bla*SPM- and *bla*NDM-1 genes responsible for metallo-β-lactamase production were horizontally transferable via plasmids and could spread rapidly to other bacteria. The first report of mobile metallo-β-lactamases was with the discovery of *P. aeruginosa* strain GN17203 from Japan in 1988. The imipenem enzymes were originally detected in Asia, but later spread to Europe, the USA, and Australia. The VIM-encoding gene was first found in Europe and emerged in other countries shortly after.

One of the limitations of this study was the small sample size of specimens, such as pus and cerebral spinal fluid, which could affect the results. However, besides the limitations, the current study had some positive outcomes.

First, we showed a relatively high frequency of *exoS* (59.2%) and *exoY* (53.9%) and a low frequency of *exoU* (10.5%) among the *P. aeruginosa* clinical isolates obtained in our area, and these frequencies were not associated with the high antibiotic resistance among the isolates. This may reflect the fact that the genes encoding the cytotoxins ExoS and ExoU were present as variable traits in *P. aeruginosa*, and their presence depended upon the disease site or background [27]. Second, it seemed that the source of bacterial isolation was associated with the trend of acquisition of specific virulence genes that may serve to cause specific infections. These results indicated the potential risk of these isolates in nosocomial infections, which needed more attention. Of course, further studies with a larger sample size and from other regions of the country were required to reach a comprehensive conclusion.

In conclusion, *P. aeruginosa*, an important causative agent of human infection, was resistant to a wide range of antimicrobial drugs. The data for the years 2010 to 2015 showed that the drug resistance was controlled in this area. Infection with a type III effector-secreting isolate has been shown to be correlated with severe disease, and the TTSS in low respiratory and systemic infections was associated with an increased mortality rate. In this study, the relative frequencies of virulence genes were different among the clinical specimens. There were nine types among the TTSS-positive strains, and there were no strains carrying all four virulence genes. The resistance data of this area showed that the drug resistance rate and the gene mutations of TTSS-negative strains were higher than those of TTSS-positive strains ($P > 0.05$), demonstrating that the TTSS-secreting isolate was not correlated with the high drug resistance in this study.

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Disclosure of conflict of interest

None.

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