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Original Article

Characterization of clinical extensively drug resistant *Pseudomonas aeruginosa* from a Chinese teaching hospital

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Abstract

Introduction: *Pseudomonas aeruginosa*, an important opportunistic pathogen, carries multiple virulence factors which contribute to its adaptation and pathogenicity. The goal of this study was to characterize the virulence factors among extensively drug-resistant *P. aeruginosa*.
Methodology: In this study, 63 non-duplicated extensively drug-resistant *P. aeruginosa* clinical isolates were collected from December 2013 to July 2015. Polymerase chain reaction (PCR) was used to analyze the homogeneity and the type III secretion system. Microtiter plate method was performed to evaluate the ability to form biofilms associated to twitching and swimming motilities.
Results: High percentage (96.8%) of isolates was sensitive to polymyxin B, while the resistance rate to other antibiotics (amikacin, aztreonam, ceftazidime, ciprofloxacin, gentamicin, imipenem, levofloxacin, meropenem, piperacillin-tazobactam) ranged from 80.9% to 100%. Enterobacterial repetitive intergenic consensus-PCR detected seven major groups with minimal genetic variation. All the isolates carried *exoT* gene, 96.8% carried *exoY*, 69.8% carried *exoS*, and 31.7% carried *exoU* gene. Biofilm formation was confirmed in all strains, out of which 41.3% formed strong biofilm. Motilities analysis showed heterogeneous diameters ranging from 6.02 to 26.09 mm for swimming and from 7.60 to 23.34 mm for twitching motilities.
Conclusions: Our findings revealed that the clinical *P. aeruginosa* isolates tested are the major invasive types in nature and multiple virulence factors were commonly carried in the extensively drug-resistant strains.

Key words: *Pseudomonas aeruginosa*; extensively drug-resistant; molecular epidemiology.

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Introduction

The opportunistic pathogen *Pseudomonas aeruginosa*, a major cause of hospital-acquired infections, is frequently isolated from severe burn wounds, implanted medical devices, urine and lungs of cystic fibrosis patients [1]. *P. aeruginosa* exploits various virulence factors, including toxins, flagella, pili and biofilm formation, to promote its pathogenicity, leading to great morbidity and mortality [1,2].

Type III secretion system (T3SS) is a predominant virulence factor for *P. aeruginosa*. Only four cytotoxins ExoS, ExoU, ExoT, and ExoY, coded by *exoS*, *exoU*, *exoT* and *exoY*, were injected into host cells, leading to inhibition of DNA synthesis, disruption of cell skeleton and enhanced resistance to phagocytosis, contributing to bacterial dissemination in the body and evasion from the immune system [3-5].

Biofilm is a complex microcolony, embedded in polysaccharide and extracellular DNA that protects and enhances its tolerance to host immune responses and antibiotics [6,7]. The bacterial flagella, which is involved in swimming motility, and type IV pili (T4P), which provides flagellar-independent movement through a solid surface called twitching motility [8,9], are essential for biofilm formation and closely linked to adhesion to human cells, evasion from stress and spreading of infection.

Little is known about the virulence gene pattern, motility and biofilm formation of extensively drug-resistant (XDR) *P. aeruginosa* in China. So the aim of this study was to analyze different virulence factors in 63 isolates of XDR *P. aeruginosa* from December 2013 to July 2015, from Xiangya Hospital, China.

Methodology

Bacterial Isolates

A total of 63 non-duplicate *P. aeruginosa* isolates were collected at Xiangya hospital, Changsha, China. The *P. aeruginosa* isolate which was not-susceptible to at least one agent in all but two or fewer antimicrobial

categories according to previous study [10], was defined as XDR and selected. The isolates were further confirmed as *P. aeruginosa* by the specific PCR described by De Vos D [11]. The isolates were stored at −70 °C for further analysis.

Bacterial isolate genotyping

Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) was performed with ERIC primers to reveal the genetic relationships among the isolates [12]. Bacterial DNA was extracted using the boiling method [13]. The PCR reactions were carried out with ABI 2720 Thermal Cycler (Applied Biosystems, Foster city, USA) in a total of 25 μL, including 1 U Taq DNA polymerase (BioTeke corporation, Beijing, China), 2.5 μL of 10 × reaction buffer, 2.0 mM Mg^{2+}, 1 μL of 0.2 mM of dNTP (BioTeke corporation, Beijing, China), 2 μL DNA template, 1 μL (10 pmol) forward and reverse primers and nuclease-free water.

The PCR procedure was as follows: initial denaturation step at 94 °C for 7 minutes, followed by 40 cycles of at 94 °C for 1 minute, at 53 °C for 1 minute, at 72 °C for 2 minutes and a final extension at 72 °C for 15 minutes. The PCR products were electrophoresed on agarose gel (1.5 %, w/v) with ethidium bromide (0.5 μg/mL) and visualized with a UV transilluminator. The dendrogram derived from this data was constructed by NTSYS-pc software with 0.5% band tolerance. Strains were defined as the same ERIC type when the coefficient was ≥ 90%. Primers used in this study were shown in Table 1.

Antibiotic susceptibility testing

Antibiotic susceptibility testing of the isolates was performed by the agar dilution method using Mueller-Hinton agar (Oxoid, Unipath, Hampshire, UK), according to the Clinical and Laboratory Standards Institute 2015 (CLSI) guidelines [14]. Ten antibiotics were tested: piperacillin-tazobactam (TZP), ceftazidime (CAZ), aztreonam (ATM), gentamicin (GEN), ciprofloxacin (CIP), levofloxacin (LEV), meropenem (MEM), imipenem (IPM), amikacin (AK), and polymyxin B (PB). Various concentrations between 0.125–256 μg/ml were tested for each antibiotic. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 (from the American Type Culture Collection) were used as quality controls.

Detection of T3SS genes

The presence of *exoS* [15], *exoY* [15], *exoU* [16] and *exoT* [17] genes were screened by PCR method. The primers and annealing temperatures were listed in Table 1. PCR products were detected using 1.2 % (w/v) agarose gel electrophoresis. The *P. aeruginosa* PAO1 reference strain was used as positive control for *exoS*, *exoY* and *exoT* genes [18]. The amplified *exoU* gene products were sequenced and compared using the Basic Local Alignment Search Tool available at the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Biofilm assay

A modified microtiter plate method was performed to evaluate the ability of the clinical *P. aeruginosa* isolates to form biofilms [19]. Briefly, overnight cultures of the isolates were adjusted to McFarland × 0.5 in fresh Luria-Bertani (LB) broth, then 100 μL of the culture was incubated in five wells of a 96-well plate at 37 °C for 24 h. The adhesive biofilms were stained with 0.3 % (w/v) crystal violet, rinsed under tap water, and resolved with 95 % ethanol. The optical density (OD) was measured at 570 nm with a microtiter plate reader (Infinite M200pro, TECAN, Salzburg, Austria). Sterile LB broth (Oxoid, Unipath, Hampshire, UK) without bacteria served as negative controls. Three independent tests were conducted.

The cutoff OD (ODc) was determined as the average OD of negative control. According to ODc, the

Table 1. Primers used for polymerase chain reaction.

Primer name	Sequence (5`-3`)	Annealing temperature
ERIC	F: ATGTAAGCTCCTGGGGATTCAC R: AAGTAAGTGACTGGGGTGAGCG	53°C
exoS	F: TCAGGTACCCGGCATTCACTACGCGG R: TCACTGCAGGTTCGTGACGTCTTTCT	55°C
exoU	F: GGGAATACTTTCCGGGAAGTT R: CGATCTCGCTGCTAATGTGTT	57°C
exoY	F: AATCGCCGTCCAACTGCATGCG R: TGTTCGCCGAGGTACTGCTC	55°C
exoT	F: TCCAAGCTTATGCGTATCGACGGTCATC R: CGTATCGATCCGAGGGGGGTGTATCTGACC	58°C

isolates were classified as no biofilm producers (OD < ODc), low producers (ODc < OD ≤ 2 × ODc), moderate producers (2 × ODc < OD ≤ 4 × ODc), and strong producers (OD > 4 × ODc).

Swimming motility assay

Swimming motility was tested as described previously [20]. A single clone of *P. aeruginosa* strains was incubated on Trypticase Soy Broth (TSB, Oxide, Unipath, Hampshire, UK) containing 0.3 % (w/v) agar at 37 °C for 24 hours using a sterile toothpick. The motility was assessed by measuring the circular zone of bacterial growth in millimeters (mm).

Twitching motility assay

Twitching motility was tested as described previously [20] with some modifications. The bacterium was stab inoculated through thin twitch plates (TSB with 1 % (w/v) agar) to the plastic plate bottom with an autoclaved toothpick. After 24 hours at 37 °C, the agar layer was gently removed and the twitching colonies were stained with crystal violet and their size measured in mm.

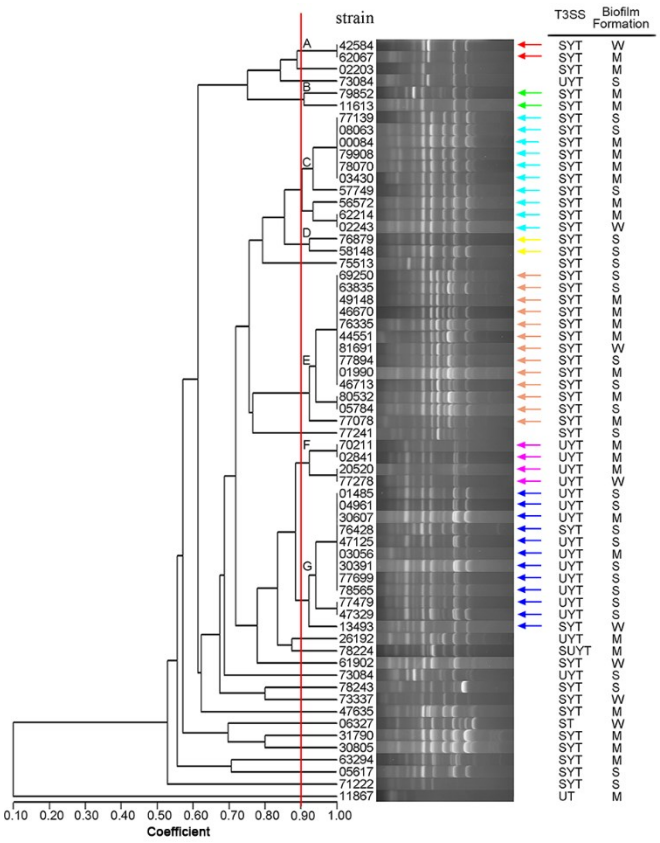
Statistical analysis

Statistical analysis was performed using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). Correlations between the T3SS genotype patterns and biofilm formation were analyzed by Pearson's chi-square or Fisher's exact tests. Differences between the biofilm formation groups were evaluated with the Kruskal–Wallis and Mann–Whitney tests. Statistical significance was defined as a p value of ≤ 0.05.

Results

A total of 63 non-duplicated *P. aeruginosa* was obtained from different areas in Xiangya hospital: 32

Figure 1. Clustering dendrogram and the T3SS gene patterns of the 63 P. aeruginosa clinical isolates.



The ERIC revealed minimal genetic variation, in which seven major clones are pointed out by the colored arrows: red (group A), green (group B), light blue (group C), yellow (group D), brown (group E), pink (group F), dark blue (group G). Red line represents Dice coefficient equal to 90%. The T3SS toxin column shows the genes carried by each isolate (i.e., exoS and exoT genes, ST; exoS, exoY and exoT genes, SYT; exoU and exoT genes, UT; exoU, exoY and exoT genes, UYT; and exoS, exoU, exoY and exoT genes, SUYT). The biofilm formation column shows biofilm forming ability for each isolate (i.e. strong, S; moderate, M; weak, W)

Table 2. Minimum inhibitory concentration (MIC) of *P. aeruginosa* clinical isolates.

Antibiotic	MIC (μg/mL)			N(%) of isolates (n = 63)		
	Range	MIC50	MIC90	S	I	R
AK	16 – >256	>256	>256	2 (3.2)	3 (4.8)	58 (92.0)
ATM	8 – >256	128	>256	3 (4.8)	9 (14.3)	51 (80.9)
CAZ	8 – >256	128	>256	3 (4.8)	2 (3.2)	58 (92.0)
CIP	4 – 64	16	64	0 (0)	0 (0)	63 (100)
GEN	32 – >256	>256	>256	0 (0)	0 (0)	63 (100)
IPM	4 – 256	32	128	0 (0)	1 (1.6)	62 (98.4)
LEV	4 – 32	16	32	0 (0)	1 (1.6)	62 (98.4)
MEM	2 – 256	16	64	1 (1.6)	4 (6.4)	58 (92.0)
PB	1 – 4	2	2	61 (96.8)	2 (3.2)	0 (0)
TZP	64/4 –> 256/4	128	>256	0 (0)	3 (4.8)	60 (95.2)

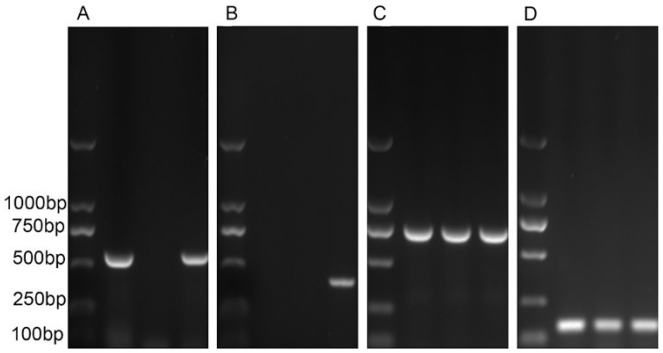
Susceptible (S), intermediate (I), resistant (R), minimum inhibitory concentration for 50% of the isolates (MIC50), minimum inhibitory concentration for 90% of the isolates (MIC90), piperacillin-tazobactam (TZP), ceftazidime (CAZ), aztreonam (ATM), gentamicin (GEN), ciprofloxacin (CIP), levofloxacin (LEV), meropenem (MEM), imipenem (IPM), amikacin (AK), and polymyxin B (PB).

(50.8%) from intensive care units, 15 (23.8%) from rehabilitation wards, five (7.9%) from burn wards, four (6.3%) from medical wards, three (4.8%) from neurosurgery wards, two (3.2%) from outpatients, and two (3.2%) from surgical wards. The clinical sample types were as follows: 49 (77.8%) from sputum and bronchial secretions, five (7.9%) from feces, four (6.3%) from wounds, three (4.8%) from blood, one (1.6%) from urine, and one (1.6%) form ascites fluid.

The resistant patterns to 10 tested antibiotics, belonging to seven categories, were shown in Table 2. All of the 63 isolates were highly resistant to AK, ATM, CAZ, GEN and TZP, with the minimum inhibitory concentration at which 90% of the isolates were inhibited (MIC_{90}) of ≥ 256 μg/mL. The resistance levels to CIP, IPM, LEV and MEM were variable. However, 61 strains were sensitive to PB and only two strains showed a MIC value of 4 μg/mL. In addition, two strains were non-susceptibility to all tested antibiotics, while 55 strains were only sensitive to a representative of one category (PB). The rest of six strains, three were sensitive to PB and ATM; three were sensitive to PB and CAZ.

The ERIC-PCR performed on 63 *P. aeruginosa* strains identified different DNA fingerprints with size that ranges from 186 bp to 972 bp. The dendrogram map (Figure 1) revealed 25 different groups. Eighteen strains had unique ERIC types while the remaining 45 strains clustered into seven groups; group C contained 10 (15.8%), group E contained 13 (20.6%), group F contained four (6.3%), group G contains 12 (19.0%) and groups A, B and D each contained two (3.2%).

Figure 2. PCR products of the T3SS genes for three isolates among 63 P. aeruginosa isolates.



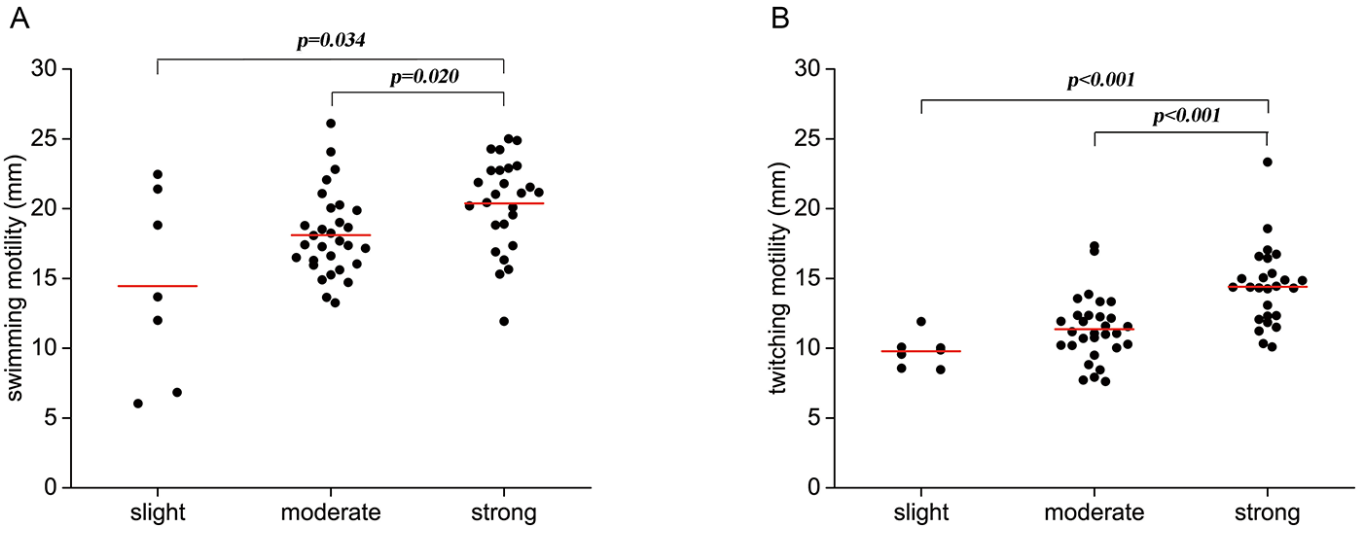
(A) Amplification of the *exoS* genes, (B) amplification of the *exoU* gene, (C) amplification of the *exoY* gene and (D) amplification of *exoT* genes.

The T3SS gene patterns showed that 63 (100%) strains carried *exoT*, while 61 strains (96.8%) carried *exoY*, 44 strains (69.8%) carried *exoS*, and 20 strains (31.7%) carried *exoU*. Only one strain (1.6%) carried both *exoS* and *exoU* (Figure 1, 2).

According to the ODc values, all the 63 isolates produced biofilms at different levels: seven strains (11.1%) formed slight biofilms, 30 strains (47.6%) formed moderate biofilms, and 26 strains (41.3%) formed strong biofilms. Furthermore, all the strains exhibited flagellar and T4P motility (diameter range: 6.02–26.09 mm, 7.60–23.34 mm, respectively).

The strong biofilm producers showed the highest level of both type of motilities ($p < 0.5$; Figure 3). There was no correlation ($p > 0.5$) between T3SS gene patterns and biofilm formation in XDR *P. aeruginosa*.

Figure 3. Distribution of flagellar swimming motilities (a) and T4P twitching motilities (b) in 63 P. aeruginosa isolates. The isolates are divided into slight, moderate and strong group according to their biofilm formation abilities.



Discussion

In this study, we identified 63 isolates of XDR *P. aeruginosa* from Xiangya Hospital over a time interval of twenty months. Antimicrobial susceptibility of the strains was test against representatives of seven categories of antibiotics, except for fosfomycin. Two strains (3.2%) were non-susceptible to all tested antibiotics; while 55 (87.3%) and six (9.5%) strains were susceptible to the representatives of only one or two categories of antibiotics, respectively. The antibacterial susceptibility test results revealed 52 strains susceptible only to polymyxin B, indicating highly resistant *P. aeruginosa* strains (Table 2), which is consistent with previous studies [21]. A wide spread of XDR *P. aeruginosa* has been disposed due to the overuse of antibiotics [22].

T3SS is a highly sophisticated virulence factor with a needle-like apparatus on the membrane, through which *P. aeruginosa* regulates host cells [23]. Four effector proteins have been identified so far and are considered as major determinants of two pathogenic types (invasive or cytotoxic). ExoS and ExoT both have GTPase-activating protein activities and ADP-ribosyl transferase activities [3]. ExoU is a potent phospholipase [4], while ExoY acts as a secreted adenylyl cyclase [5].

Our results identified *exoY* and *exoT* gene as the most prevalent genes in *P. aeruginosa*, which in agreement with other study [15]. Nevertheless, the *exoU* prevalence rate in our study was 31.7%, a finding similar to a *post hoc* analysis of *P. aeruginosa* bloodstream infections where a rate of 21% was reported by Carmen Peña [24]. However, previous studies on multi-drug resistant *P. aeruginosa* infections on patients with diabetic foot or burn conditions showed *exoU* prevalence rates of 69.8% and 64.5%, respectively, which were much higher than our own [25,26]. The distinct different prevalence rate for *exoU* gene suggests that the carriage of T3SS genes is associated with the disease sites.

Biofilm serves as a significant virulence factor by providing a shelter against antibiotics and host immune responses [7]. Lakshmi found all of *P. aeruginosa* isolated from endophthalmitis can form biofilm [27]. However, Heydari demonstrated only 43.5% *P. aeruginosa* from burn patients produced biofilm [28]. Our results have shown that all the isolates produced biofilm at different levels. In current study, the strains showed high diversity in flagellar swimming and T4P twitching motility. Statistical analyses showed the strong biofilm-formation group had higher motilities than the moderate and weak biofilm-forming groups, indicating that swimming and twitching motilities are correlated with biofilm formation (Figure 3), but not absolute necessity. Based on previous study, flagellar and T4P aid in the initial attachment and biofilm formation [29]. Interestingly, further analysis found no correlation between biofilm formation and T3SS gene patterns, which is inconsistent with the results of Choy where the authors found a strong correlation between *exoU* and biofilm formation in keratitis infections [30]. Probably because most of our strains (77.8%) were collected from sputum and bronchial secretion, this difference may suggest that T3SS genotype and biofilm formation are influenced by distinct infection sites and play an important role in the pathogenesis of specific infections for *P. aeruginosa*.

ERIC-PCR is a fast typing method and has been widely used in epidemiological studies in *P. aeruginosa*. Based on the DNA bands, seven major groups, accounting for 45 strains, were detected, implying that in this particular hospital some major genetic types of XDR *P. aeruginosa* were spreading. Similar genetic relationship was also reported previously [31], in which only three strains exhibited polymorphism among 15 multi-drug resistant *P. aeruginosa*, indicating minor genetic variation in the XDR *P. aeruginosa* in the collection period.

Conclusion

To conclude, we characterized 63 isolates of *P. aeruginosa* and confirmed a predominant type of extensively drug-resistant invasive strains spreading in the hospital. More importantly, *P. aeruginosa* employs swimming and twitching motilities, which are correlated to biofilm formation, which make drug treatment more complex.

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