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.

*J Infect Dev Ctries* 2018; 12(10):835-841. doi:10.3855/jidc.10743

(Received 01 August 2018 – Accepted 03 October 2018)

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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 \textit{P. aeruginosa} by the specific PCR
described by De Vos D [11]. The isolates were stored at
−70 °C for further analysis.

\textbf{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\textsuperscript{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
\textsc{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.

\textbf{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), ciprofloxacine (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.
\textit{Escherichia coli} ATCC 25922 and \textit{P. aeruginosa}
ATCC 27853 (from the American Type Culture
Collection) were used as quality controls.

\textbf{Detection of T3SS genes}

The presence of \textit{exoS}[15], \textit{exoY}[15], \textit{exoU}[16] and \textit{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 \textit{P. aeruginosa} PAO1
reference strain was used as positive control for \textit{exoS},
\textit{exoY} and \textit{exoT} genes [18]. The amplified \textit{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).

\textbf{Biofilm assay}

A modified microtiter plate method was performed
to evaluate the ability of the clinical \textit{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

\begin{table}
\centering
\caption{Primers used for polymerase chain reaction.}
\begin{tabular}{|l|l|l|}
\hline
\textbf{Primer name} & \textbf{Sequence (5’-3‘)}  & \textbf{Annealing temperature} \\
\hline
ERIC       & F: ATGTAAGCTCTCGGGATGATCAC  \\
            & R: AAGTAAGTGAAGGGGTTAGG    & 53°C  \\
exoS       & F: TCAAGGTACCCGGATGTTAGG    & 55°C  \\
            & R: TCACTTGCAAGTTTCCCTAAG    &      \\
exoU       & F: GGGAATACCTTTCCCGGAGTT    & 57°C  \\
            & R: CGATCTCGTCTGTATGTT       &      \\
exoY       & F: AATCGGCCGTCACCGTATGCG    & 55°C  \\
            & R: TGTTCGCGAGGTAGCTGCT      &      \\
exoT       & F: TCCAAGGTTGCTACGTGTCAT    & 58°C  \\
            & R: CGTATCGATCGAGGGTTGAT      &      \\
\hline
\end{tabular}
\end{table}
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

| Antibiotic | MIC (μg/mL) | N(%) of isolates (n = 63) |
|------------|-------------|--------------------------|
|            | Range       | MIC50 | MIC90 | S  | I  | R  |
| AK         | 16–>256     | >256  | >256  | 2  | 3  | 58 |
| ATM        | 8–>256      | 128   | >256  | 3  | 9  | 51 |
| CAZ        | 8–>256      | 128   | >256  | 3  | 2  | 58 |
| CIP        | 4–64        | 16    | 64    | 0  | 0  | 63 |
| GEN        | 32–>256     | >256  | >256  | 0  | 0  | 63 |
| IPM        | 4–256       | 32    | 128   | 0  | 1  | 62 |
| MEM        | 4–32        | 16    | 32    | 0  | 1  | 62 |
| PB         | 1–4         | 2     | 2     | 61 | 2  | 0  |
| TZP        | 64/4–>256/4 | 128   | >256  | 0  | 3  | 60 |

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%) from 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$\text{_{90}}$) 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%).

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.
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 adenyl 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.

Acknowledgements

We thank Dr Neng Li, School of Basic Medical Sciences, Fujian Medical University, for providing the *P. aeruginosa* PAO1 strain. We thank all staff in the Microbiology Department of Xiangya Hospital for their assistance with bacterial collection. This work was supported by the Hunan Development and Reform Investment (2012) No.1493 from the Development and Reform Commission of the Hunan Province, a grant (14JJ7003) from the Natural Science Foundation of the Hunan Province, and the Hunan Development and Reform Investment (2014) No.658 from Development and Reform Commission of the Hunan Province.
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**Conflict of interests:** No conflict of interests is declared.