Original Article

Characterization of Imipenem Unsusceptible *Pseudomonas Aeruginosa* Isolates from Inpatients without Carbapenem Treatment

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Objective To identify the risk factors for imipenem resistance development and transmission of clinical *Pseudomonas aeruginosa* isolates.

Methods Thirty-seven imipenem unsusceptible *Pseudomonas aeruginosa* isolates collected from patients in absence of carbapenem treatment were characterized by antimicrobial susceptibility test, pulsed field gel electrophoresis (PFGE) and carbapenem resistant mechanism analysis.

Results Before the collection of imipenem unsusceptible *Pseudomonas aeruginosa* isolates, the average time of patients treated with more than one antimicrobial (20.0 ± 9.5 days, $n = 16$) was significantly longer than those treated with only one antimicrobial (12.6 ± 4.4 days, $n = 21$; t -test, Welch, $t = -2.9004$, $P < 0.01$). And 32 isolates showed resistance to more than 3 classes of antimicrobials. Six PFGE clusters were identified and 26 isolates were grouped into one dominant cluster (C2). An ISpa1328 sequence insertion in *oprD* was detected in 33 isolates and the function of efflux was observed in all 37 isolates in the presence of a wide spectrum efflux inhibitor.

Conclusions Our data demonstrated that exposure to non-carbapenem drug classes, especially fluoroquinolones and β -lactams, may be important risk factors for the spread of carbapenem resistant *Pseudomonas aeruginosa*.

Key words: *Pseudomonas aeruginosa*; Imipenem resistance; Carbapenem

P*seudomonas aeruginosa* (*P. aeruginosa*) is one of the leading nosocomial pathogens associated with a high morbidity and mortality rate worldwide.¹ As it has remarkable ability of acquiring resistance mechanisms to multiple groups of antimicrobial agents, the increasing prevalence of nosocomial infections produced by multidrug-resistant *P. aeruginosa* isolates, including isolates with resistance to broad-spectrum-lactams, aminoglycosides and fluoroquinolones, has severely compromised the selection of appropriate treatment.² Carbapenems are currently one of the empirical options to treat multidrug-resistant *P. aeruginosa* infections.³ However, carbapenem resistance is also sharply increasing worldwide and jeopardizes the few therapeutic options in multidrug-resistant *P. aeruginosa* infection treatment.⁴

Carbapenem resistance in *P. aeruginosa* is considered to be the result of an interplay of low permeability, activity of the efflux pump, activity of an inducible or derepressed chromosomal β -lactamase (Amp C) as well

as metalloenzymes.¹ The emergence and transmission of carbapenem resistant isolates have been related to the use of carbapenems.⁵ Recently, the emergence of carbapenem resistance in *P. aeruginosa* isolates from a patient in the absence of carbapenem therapy has attracted attention to the factors contributing to the emergence and spread of carbapenem resistant *P. aeruginosa*.⁶ The effect of using other antimicrobials in the emergence and transmission of carbapenem resistant isolates was not clear. Clinical studies are needed to identify risk factors for imipenem resistance development and the characteristics of *P. aeruginosa* isolates that have emerged without carbapenem treatment.

In this study, 37 imipenem resistant *P. aeruginosa* isolates recovered from individual inpatients in absence of carbapenem treatment were analyzed. Risk factors of imipenem resistance emergence and imipenem resistance mechanisms in these isolates were characterized.

MATERIALS AND METHODS

Bacterial strains

In total, 37 non-duplicate imipenem unsusceptible *P.*

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aeruginosa isolates [imipenem minimum inhibitory concentration (MIC) = 8 mg/L (n = 5) or 16 mg/L (n = 32)] recovered from the airway secretion sample of individual patient in 3201 Hospital (Hanzhong, China) were included in this study. And all these imipenem resistant isolates were recovered for the first time from these patients after treatment. The 3201 Hospital is a 1000-bed, university-affiliated medical center that is located in southwest of China and is one of the sentinel hospitals in the network of National Center for Surveillance of Antimicrobial Resistance. These isolates were collected from neurosurgery (18 isolates), respiratory medicine (10 isolates), hepatobiliary surgery (4 isolates) and other sections (5 isolates) from July 2007 to March 2008 (Figure 1). All *P. aeruginosa* isolates were identified by the API 32GN system (BioMerieux, France). *P. aeruginosa* ATCC27853, IMP-1 or VIM-2 producing *P. aeruginosa* isolates and *P. aeruginosa* PAO1 were taken as control strains in susceptibility testing and β -lactamase assays.

Antimicrobial susceptibility testing

The minimal inhibitory concentrations (MICs) to 11 antimicrobials were measured via broth microdilution method for all 37 *P. aeruginosa* isolates, including amikacin, colistin, cefepime, cefotaxime, ceftazidime, ciprofloxacin, imipenem, meropenem, piperacillin-tazobactam, trimethoprim-sulfamethoxazole and tetracycline. All susceptibility results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) interpretive standards.⁷ All 37 isolates were screened for metallo- β -lactamase production by E-test (imipenem/EDTA) (AB BIODISK, Sweden) method according to the manufacture's instructions.

Pulsed-field gel electrophoresis (PFGE)

All 37 imipenem unsusceptible *P. aeruginosa* *Stenotrophomonas maltophilia* isolates were analyzed by PFGE to determine DNA fingerprinting profiles resulting from digestion by restriction enzymes *Spe* I according to the reference method with minor modifications.⁸ Briefly, bacteria were grown and suspended in cell suspension buffer (100 mmol/L Tris HCl, 100 mmol/L EDTA; pH 8.0) and adjusted to an optical density of 0.52 to 0.56 MCF. The cell suspension (200 μ l) was mixed with 10 μ l proteinase K (20 mg/ml) and an equal volume of melted 1% agarose. After solidification, the agarose plugs were treated with proteinase K for 2 hours in a water bath at 54°C with agitation at 180 rpm. The plugs were washed and treated with 50 U *Spe* I (New England Biolab, Beijing LTD) for two hours in a water bath at 37°C. The resulting DNA fragments were resolved by electrophoresis in a 1% agarose gel using a Chef Mapper (Bio-Rad, Beijing, China) with pulse times of 2.16 s to 63.8 s.

The gel was stained with ethidium bromide, and DNA bands were visualized with a UV transilluminator. The interpretation of the PFGE patterns was performed with BioNumerics software (Applied Maths, Belgium) by the Dice Similarity coefficient. The tree indicating relative genetic similarity was constructed on the basis of the Unweighted Pair Group Method of Averages (UPGMA), with position tolerance of 1%. Clusters were defined as DNA patterns sharing $\geq 85\%$ similarity (C1, C2, C3, ...).

PCR amplification and DNA sequence analysis

Metallo- β -lactamase encoding genes, including blaIMP, blaVIM, blaGIM, blaSPM and blaSIM were amplified by PCR as previously described.⁹⁻¹³ Other β -lactamase encoding genes, including blaTEM, blaOXA and blaSHV were also screened.^{14,15} PCR amplification of oprD sequence was performed as previously reported.¹⁶ The expression of ampC was determined by real-time reverse transcriptase PCR (RT-PCR) as previously described and compared to the control strain PAO1. More than 10-fold ampC expression was considered as over-expression.¹⁷ All purified PCR products were sequenced and the sequences obtained were analyzed by Sequencher 4.6 software (Gene Codes Corporation, USA).

Efflux analysis

To investigate the contribution of efflux in imipenem resistance, the MIC of imipenem was determined by broth microdilution method in the presence or absence of the wide-spectrum efflux inhibitor phenyl-arginine- β -naphthylamide (PA β N, 50 mg/L, Sigma-Aldrich).

Statistical methods

The antimicrobial treatment time of the patients treated with one or more antimicrobials was compared by *t*-test with Epi Info 6 software (www.cdc.gov/epiinfo/).

RESULTS

Epidemiological data of *P. aeruginosa* isolates

For each patient, the average recovery time of the first imipenem unsusceptible isolate was 15.8 ± 7.9 days (n = 37) after the patients were treated with antimicrobials. Twenty-one *P. aeruginosa* isolates were isolated from patients treated with one antimicrobial, with the average course of therapy as 12.6 ± 4.4 days; 16 isolates were isolated from patients treated with two to four antimicrobials, with the average course of therapy as 20.0 ± 9.5 days, which was significantly longer than those treated with one antimicrobial (*t*-test, Welch, *t* = -2.9004, *P* < 0.01). Among the 37 patients, 26 were treated with β -lactams, including ceftizoxime (n = 10), ceftazidime (n = 8), cefotaxime (n = 4),

azlocillin (n = 2), cefoperazone (n = 2), cefepime (n = 1) and piperacillin-tazobactam (n = 1). Twenty-two patients were treated with fluoroquinolones, including levofloxacin (n = 14), gatifloxacin (n = 6) and ciprofloxacin (n = 2). The amount of patients treated with β -lactams only and fluoroquinolones only were 12 and 10, respectively, and 11 patients received both fluoroquinolones and β -lactams for treatment. Total of 8 patients were treated with gentamicin (n = 7) or azithromycin (n = 1).

Resistance profiles of these isolates

All 37 *P. aeruginosa* isolates were susceptible to colistin and cefepime based on the CLSI breakpoints, but 32 isolates showed MIC to cefepime as either 8 mg/L (n = 16) or 16 mg/L (n = 16) and 33 isolates showed MIC to meropenem either as 4 mg/L (n = 25) or 8 mg/L (n = 8). Resistance to tetracycline was the most common (100%, 37/37) followed by trimethoprim-sulfamethoxazole (86.5%, 32/37) and amikacin (85.1%, 32/37). The prevalence of resistance to cefotaxime

and ceftazidime were 16/37 and 3/37. Among the 37 isolates, 13 isolates were resistant to ciprofloxacin, and 23 isolates showed MIC to ciprofloxacin either as 1 mg/L (n = 5) or 2 mg/L (n = 18). Total of 11 unique antimicrobial resistant profiles were identified among these 37 isolates and 32 isolates showed resistance to more than 3 classes of antimicrobials (Figure 1).

PFGE and antimicrobial resistance mechanisms analysis

In total, six PFGE clusters were identified among 37 *P. aeruginosa* imipenem unsusceptible isolates and 26 isolates were grouped into one dominant cluster (C2). All isolates were phenotypically negative for metallo- β -lactamase by E-test method, but blaIMP-9 was detected in isolate 15 by PCR and further confirmed by sequence analysis. An ISpa1328 sequence insertion at position 117 nucleotides downstream of the oprD translational start codon was found in 33 isolates. The sequences flanking the IS elements represent the direct repeat sequence of the oprD (8 bp). blaOXA-10 was detected

Figure 1. Dendrogram of patterns obtained by pulsed-field gel electrophoresis (PFGE) of 37 imipenem unsusceptible *Pseudomonas aeruginosa* isolates from inpatients without carbapenem treatment.

Notes: AMK, denotes amikacin; CTX, cefotaxime; CAZ, ceftazidime; CIP, ciprofloxacin; IMP, imipenem; MEM, meropenem; TZP, piperacillin-tazobactam; SXT, trimethoprim/sulfamethoxazole and TET tetracycline.

in 36 isolates, blaTEM-1 was detected in 33 isolates (Figure 1). All 37 isolates were negative for blaGIM, blaSHV, blaSPM, blaSIM and blaVIM by PCR. In the presence of PA β N, the susceptibility to imipenem was increased 2- (5 isolates), 4- (19 isolates) and 8-fold (13 isolates), respectively; susceptibility to meropenem was increased 2- (3 isolates), 4- (2 isolates), 8- (30 isolates) and 16-fold (2 isolates), respectively. AmpC over-expression was identified in 2 isolates (isolates 40 and 46).

DISCUSSION

The identification of risk factors that contribute to the emergence and transmission of carbapenem-resistant *P. aeruginosa* isolates is critical for nosocomial infection prevention and clinical treatment. Previous studies have recognized different risk factors that are related to the emergence and transmission of carbapenem resistant *P. aeruginosa* isolates, including prior carbapenem exposure,^{18,19} ICU stay,⁴ patient-to-patient transmission,²⁰ etc. Recently, a case study showed chronic exposure to unrelated drug classes might be an important determinant for the emergence of carbapenem resistance in *P. aeruginosa*.⁶ Our data showed that the use of non-carbapenem antimicrobials, especially fluoroquinolones and cephalosporins could select *P. aeruginosa* unsusceptible to carbapenem. But more than 2 classes of antimicrobial application could significantly delay the colonization of isolates unsusceptible to imipenem. These data suggested the emergence of carbapenem resistance should not only be considered among patients receiving carbapenem treatment but also among patients absent of carbapenem treatment.

In this study, 32 out of 37 *P. aeruginosa* isolates unsusceptible to imipenem showed resistance to more than 3 classes of antimicrobials which further emphasized the remarkable ability of these bacteria to acquire resistance mechanisms of multiple groups of antimicrobial agents.¹ The multi-drug resistance phenotypes of these isolates further verified that other antimicrobials application were risk factors for the colonization of carbapenem resistant isolates. Most isolates showed resistance or intermediate susceptibility to fluoroquinolones and the third generation cephalosporins which were the preferred antimicrobials to *P. aeruginosa* infections in local hospitals. Since non-carbapenem antimicrobials could also select imipenem unsusceptible isolates and accelerate their transmission in local hospitals, targeted approaches should be developed to stop or slow down the transmission of carbapenem resistance in absence of carbapenem treatment and to protect carbapenem clinical efficiency.

In this study, 6 PFGE clusters were identified among 37 imipenem unsusceptible isolates which demonstrated that different *P. aeruginosa* clones unsusceptible to imipenem might exist in this hospital. But an identical ISPa1328 sequence was detected in oprD at the same insertion position among the 32 isolates. Loss of OprD porin has been widely reported as a typical mechanism for imipenem resistance and reduced susceptibility to meropenem in *P. aeruginosa*.^{21,22} ISPa1328 was first detected in 4 clinical isolates from Texas of the United States and also reported in a recent study of Chinese isolates.^{23,16} Why this insertion sequence was so widely distributed among *P. aeruginosa* isolates of different genotypes was still not clear. Extensive studies should be conducted to characterize the transposition mechanism of this transposon among clinical isolates.

In previous studies, metallo-beta-lactamases have been widely reported among imipenem resistant *P. aeruginosa* isolates.²⁴ But in this study, all 37 isolates were phenotypically negative for metallo-beta-lactamases and only one isolate was positive by PCR, the metallo-beta-lactamases gene was not well expressed even in this isolate as shown by the E-test. Besides OprD porin inactivation, inhibition of efflux by PA β N in these imipenem unsusceptible isolates could also increase the susceptibility to carbapenems. These data indicated efflux was another carbapenem resistance mechanism widely distributed in these isolates, such as mexEF-oprN overexpression as previously reported.²⁵

We reported the emergence and spread of carbapenem resistance among patients never treated with carbapenem were related to the use of fluoroquinolones or β -lactams, especially the treatment of one antimicrobial. Prudent antimicrobial selection policies combined with good infection control practices should be adopted to stop the development and spread of carbapenem resistance, and to ensure that these important agents could maintain their efficiency in the therapy of *P. aeruginosa* infections.

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Disclosure Statement

No competing financial interests exist.

REFERENCES

1. Strateva T, Yordanov D. Pseudomonas aeruginosa - a phenomenon of bacterial resistance. J Med Microbiol 2009;58:1133-1148.
2. McGowan JE Jr. Resistance in nonfermenting gram-negative bacteria: multidrug resistance to the maximum. Am J Infect Control 2006;34:s29-s37; discussion s64-s73.

3. Mesaros N, Nordmann P, Plesiat P, Roussel-Delvallez M, Van Eldere J, Glupczynski Y, et al. *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millennium. *Clin Microbiol Infect* 2007;13:560-578.
4. Furtado GH, Bergamasco MD, Menezes FG, Marques D, Silva A, Perdiz LB, et al. Imipenem-resistant *Pseudomonas aeruginosa* infection at a medical-surgical intensive care unit: risk factors and mortality. *J Crit Care* 2009;24:625-629-614.
5. Shigemi A, Matsumoto K, Yaji K, Shimodozono Y, Takeda Y, Miyanohara H, et al. Correlation between meropenem and doripenem use density and the incidence of carbapenem-resistant *Pseudomonas aeruginosa*. *Int J Antimicrob Agents* 2009;34:589-591.
6. Wolter DJ, Acquazzino D, Goering RV, Sammut P, Khalaf N, Hanson ND. Emergence of carbapenem resistance in *Pseudomonas aeruginosa* isolates from a patient with cystic fibrosis in the absence of carbapenem therapy. *Clin Infect Dis* 2008;46:e137-141.
7. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing: Seventeenth Informational Supplement M100-S19. CLSI, Wayne, PA, USA:2009.
8. Pitout JD, Chow BL, Gregson DB, Laupland KB, Elsayed S, Church DL. Molecular epidemiology of metallo-beta-lactamase-producing *Pseudomonas aeruginosa* in the Calgary Health Region: emergence of VIM-2-producing isolates. *J Clin Microbiol* 2007;45:294-298.
9. Castanheira M, Toleman MA, Jones RN, Schmidt FJ, Walsh TR. Molecular characterization of a beta-lactamase gene, blaGIM-1, encoding a new subclass of metallo-beta-lactamase. *Antimicrob Agents Chemother* 2004;48:4654-4661.
10. Lauretti L, Riccio ML, Mazzariol A, Cornaglia G, Amicosante G, Fontana R, et al. Cloning and characterization of blaVIM, a new integron-borne metallo-beta-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. *Antimicrob Agents Chemother* 1999;43:1584-1590.
11. Senda K, Arakawa Y, Ichihama S, Nakashima K, Ito H, Ohsuka S, et al. PCR detection of metallo-beta-lactamase gene (blaIMP) in gram-negative rods resistant to broad-spectrum beta-lactams. *J Clin Microbiol* 1996;34:2909-2913.
12. Toleman MA, Simm AM, Murphy TA, Gales AC, Biedenbach DJ, Jones RN, et al. Molecular characterization of SPM-1, a novel metallo-beta-lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance programme. *J Antimicrob Chemother* 2002;50:673-679.
13. Tsakris A, Pournaras S, Woodford N, Palepou MF, Babini GS, Douboyas J, et al. Outbreak of infections caused by *Pseudomonas aeruginosa* producing VIM-1 carbapenemase in Greece. *J Clin Microbiol* 2000;38:1290-1292.
14. Bert F, Branger C, Lambert-Zechovsky N. Identification of PSE and OXA beta-lactamase genes in *Pseudomonas aeruginosa* using PCR-restriction fragment length polymorphism. *J Antimicrob Chemother* 2002;50:11-18.
15. Haeggman S, Lofdahl S, Paauw A, Verhoef J, Brisse S. Diversity and evolution of the class A chromosomal beta-lactamase gene in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2004;48:2400-2408.
16. Wolter DJ, Hanson ND, Lister PD. Insertional inactivation of oprD in clinical isolates of *Pseudomonas aeruginosa* leading to carbapenem resistance. *FEMS Microbiol Lett* 2004;236:137-143.
17. Quale J, Bratu S, Gupta J, Landman D. Interplay of efflux system, ampC, and oprD expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical isolates. *Antimicrob Agents Chemother* 2006;50:1633-1641.
18. Fujimura S, Nakano Y, Sato T, Shirahata K, Watanabe A. Relationship between the usage of carbapenem antibiotics and the incidence of imipenem-resistant *Pseudomonas aeruginosa*. *J Infect Chemother* 2007;13:147-150.
19. Lepelletier D, Cady A, Caroff N, Marraillac J, Reynaud A, Lucet JC, et al. Imipenem-resistant *Pseudomonas aeruginosa* gastrointestinal carriage among hospitalized patients: risk factors and resistance mechanisms. *Diagn Microbiol Infect Dis* 2010;66:1-6.
20. Johnson JK, Smith G, Lee MS, Venezia RA, Stine OC, Nataro JP, et al. The role of patient-to-patient transmission in the acquisition of imipenem-resistant *Pseudomonas aeruginosa* colonization in the intensive care unit. *J Infect Dis* 2009;200:900-905.
21. Rodriguez-Martinez JM, Poirel L, Nordmann P. Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2009;53:4783-4788.
22. Walsh F, Amyes SG. Carbapenem resistance in clinical isolates of *Pseudomonas aeruginosa*. *J Chemother* 2007;19:376-381.
23. Wang J, Zhou JY, Qu TT, Shen P, Wei ZQ, Yu YS, et al. Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Chinese hospitals. *Int J Antimicrob Agents* 2010;35:486-491.
24. Walsh TR, Toleman MA, Poirel L, Nordmann P. Metallo-beta-lactamases: the quiet before the storm? *Clin Microbiol Rev* 2005;18:306-325.
25. Kohler T, Epp SF, Curty LK, Pechere JC. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. *J Bacteriol* 1999;181:6300-6305.