J Infect Chemother xxx (2017) 1–8

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original Article

Association of the *exoU* genotype with a multidrug non-susceptible phenotype and mRNA expressions of resistance genes in *Pseudomonas aeruginosa*☆

Iichiro Takata, Yuka Yamagishi, Hiroshige Mikamo*

Department of Clinical Infectious Diseases, Aichi Medical University Graduate School of Medicine, Aichi, Japan

ARTICLE INFO

Article history:
Received 27 May 2017
Received in revised form
10 August 2017
Accepted 31 August 2017
Available online xxx

Keywords:
Pseudomonas aeruginosa
Efflux pump
Virulence factor
Multidrug resistance

ABSTRACT

The increased prevalence of the virulence factor *exoU* + genotype among multidrug-resistant *Pseudomonas aeruginosa* has been previously reported. However, the genes that are related to the multidrug resistance of the *exoU* + genotype strain have not been analyzed and remain to be elucidated. The objective of this study was to analyze the correlations between virulence factors and resistance genes. The *exoU* + genotype was frequently found in carbapenem and fluoroquinolone non-susceptible strains. The *imp* carbapenemase genotype, the quinolone-resistance-determining region mutation in GyrA and ParC and the defective mutation in OprD were not frequently found in the *exoU* + genotype and carbapenem and fluoroquinolone non-susceptible strains. On the other hand, *mexY* and *ampC* mRNA overexpressing strains were more frequently found in the *exoU* + genotype and carbapenem and fluoroquinolone non-susceptible strains. Moreover, sequence type 235, a high risk clone of multidrug-resistant *P. aeruginosa*, was prevalent among the *exoU* + genotype and carbapenem and fluoroquinolone non-susceptible strains. ExoU is highly virulent protein, and the overexpression of efflux pumps and AmpC β-lactamase induce a multidrug-resistant phenotype. Therefore, the increased prevalence of *P. aeruginosa* strains with an *exoU* + genotype and the overexpression of efflux pumps and AmpC β-lactamase are likely to make *P. aeruginosa* infections difficult to treat. An understanding of the prevalence of both the *exoU* + genotype and the mRNA overexpression of resistance genes may help to select empirical therapy for the treatment of nosocomial infections caused by *P. aeruginosa*.

1. Introduction

Pseudomonas aeruginosa is a gram-negative pathogen that is associated with serious nosocomial infections. Infection with *P. aeruginosa* is a frequent cause of death among patients with ventilator-associated pneumonia [1].

One of the reasons for the poor clinical outcomes of *P. aeruginosa* infections is thought to be virulence factors, especially the type III secretion system (TTSS) [2]. Four toxins (ExoS, ExoT, ExoU and ExoY) can be secreted via the TTSS. Almost all strains express the ExoT and ExoY toxins. However, the ExoS and ExoU toxins are mutually exclusive in most strains, and these toxins have different prevalence depending on the source of the isolation and the patient's background [3]. Associations between these toxins and the virulence of clinical isolates have been reported [4,5]. A retrospective analysis of *P. aeruginosa* bacteremia showed that the expression of TTSS-associated toxins was correlated with the 30-day mortality rate [6]. The high virulence of TTSS-associated toxins in a murine infection model [7,8] supports the clinically observed outcomes.

One of the other risk factors for mortality in patients infected with *P. aeruginosa* is multidrug resistance [9]. The rate of multidrug resistance especially to fluoroquinolones and carbapenems in *P. aeruginosa* clinical isolates has increased and is becoming a major issue for nosocomial infection [10,11]. The mechanisms of resistance to fluoroquinolones are recognized as target-site gene mutations, the increased production of multidrug efflux pumps, and enzyme protection and modification [12,13]. On the other

☆ All authors meet the ICMJE authorship criteria.

* Corresponding author. Department of Clinical Infectious Diseases, Aichi Medical University Graduate School of Medicine, 1-1 Yazakokarimata, Nagakute, Aichi, Japan.
E-mail address: mikamo@aichi-med-u.ac.jp (H. Mikamo).

http://dx.doi.org/10.1016/j.jiac.2017.08.018
1341-321X/

hand, the mechanisms of resistance to carbapenems are recognized as target-site gene mutations, the increased production of multidrug efflux pumps, the mutation of porins, and hydrolysis by carbapenemase [14,15]. Among these mechanisms, RND-type efflux pumps exist in gram-negative bacteria and excrete many antibiotics [16].

The correlation between the prevalence of TTSS toxin genes and multidrug resistance in clinical isolates of *P. aeruginosa* has been previously investigated [5,17]. The increased prevalence of strains that overexpress the mRNA of RND-type efflux pumps and *ampC* β-lactamase among resistant *P. aeruginosa* have also been reported [18]. However, these correlations were investigated separately, and the correlation between the TTSS genotype and resistance genes, especially those related to multidrug resistance such as multidrug efflux pumps, have not yet been investigated.

In this study, the correlations between carbapenem and fluoroquinolone non-susceptibility and the TTSS genotypes or mRNA overexpression of resistance genes were analyzed. Furthermore, the correlations between the *exoU* + genotype and resistance genes related to multidrug non-susceptibility were analyzed.

2. Materials and methods

2.1. Bacterial isolates

The susceptibility to antimicrobial agents of a total of 764 *P. aeruginosa* clinical isolates obtained from patients at Aichi Medical University Hospital in Japan between 2012 and 2014 were tested (data not shown). One hundred twenty isolates (15.7%) were non-susceptible to meropenem, imipenem and ciprofloxacin, and 440 isolates (57.6%) were susceptible to meropenem, imipenem and ciprofloxacin. Furthermore, the strains isolated from the same patients were excluded so that 50 non-susceptible isolates and the equal number of susceptible isolates were analyzed.

2.2. Antimicrobial susceptibility testing

Susceptibility testing was performed by measuring the MIC of meropenem, imipenem, ciprofloxacin, ceftazidime, tazobactam/piperacillin and amikacin. Meropenem and imipenem were purchased from U.S. Pharmacopeial Convention (USA). Ciprofloxacin was purchased from MP Biomedicals, LLC (USA). Tazobactam was purchased from LKT Laboratories, Inc. (USA). Amikacin, piperacillin and ceftazidime were purchased from Sigma-Aldrich Japan (Japan).

The MICs were determined using the microdilution method, according to the Clinical and Laboratory Standards Institute (CLSI) guideline [19].

2.3. Genotyping of TTSS genes and β-lactamase genes by PCR

Genotyping of the *exoS*, *exoT*, *exoU* and *exoY* genes was performed using a PCR method as reported previously [20]. Genotyping of the bla_{VIM}, bla_{IMP}, bla_{KPC}, bla_{GES} and bla_{NDM} genes was performed using a PCR method, as reported previously [21]. The primers used in this study are shown in Table 1. Bacterial cells were dissolved in Gene releaser® reagent (BioVentures, Inc., USA) for genotyping of TTSS and TE for genotyping of β-lactamase genes and treated at 95 °C for 10 min to prepare template chromosomal DNA. PCR products were visualized using gel electrophoresis on a 3% (w/v) agarose gel.

2.4. RNA isolation and reverse transcription to cDNA

Overnight cultures were suspended in Mueller–Hinton II broth and adjusted to give a turbidity equivalent to that of a 0.5 McFarland standard. The cells were incubated in a shaking water bath at 35 °C for 120 min before the addition of twice the amount of RNA protect bacteria reagent (Qiagen, Japan) and lysozyme (Wako Pure Chemical Industries, Ltd., Japan) to a final concentration of 1 mg/mL. The total RNA was isolated using the RNeasy mini kit (Qiagen, Japan) according to the manufacturer's protocol. Reverse transcription to cDNA from the total RNA was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Japan) with random hexamers, according to the manufacturer's protocol.

2.5. Quantitative RT-PCR

The relative mRNA expression levels of *mexB, mexD, mexF, mexY* and *ampC* were determined using a real-time PCR method. The primers and probes used in this study are shown in Table 1. The probes were synthesized by Applied Biosystems Japan. A total of 1.5 μL of cDNA was used in a final volume of 15 μL reaction, containing 500 nM of each primer, 50 nM of each probe, and 1 × TaqMan® universal master mix (Applied Biosystems Japan, Japan). Quantitative RT-PCR was performed using Applied Biosystems 7500 Fast (Applied Biosystems Japan, Japan). The reactions were performed in triplicate on a 96-well plate. The *rpsL* housekeeping gene was used as an internal standard. *P. aeruginosa* PAO1 was used as a control strain. Overexpression was defined as an expression level that was at least 3-fold higher than that of *P. aeruginosa* PAO1 for *mexB* mRNA and at least 10-fold higher than that of *P. aeruginosa* PAO1 for *mexD*, *mexF*, *mexY* and *ampC* mRNA, as reported previously [22].

2.6. Sequencing of oprD and quinolone-resistance-determining region (QRDR) of gyrA and parC

The primers used in this study are shown in Table 2. The amplification of QRDR of *gyrA* and *parC* was performed using a PCR method, as reported previously [23,24]. The amplification of *oprD* was performed using the following protocol: 2 min at 95 °C, then 30 cycles of 30 s at 95 °C, 30 s at 55 °C, 60 s at 72 °C, and a final extension step for 2 min at 72 °C. PCR products were sequenced using the Applied Biosystems 3730/3730xl DNA analyzers sequencing system (Applied Biosystems Japan, Japan). The coding region of *oprD* and QRDR of *gyrA* and *parC* gene were compared with that of the reference strain *P. aeruginosa* PAO1 (GenBank accession no. AE004091.2). The correlation between the *exoU* + genotype and amino acid mutation in QRDR of GyrA and ParC was evaluated as previously reported method [17]. The defective mutation in OprD was defined as previously reported method [25].

2.7. Multilocus sequence typing (MLST)

MLST was performed as described previously [26]. The primers used in this study are shown in Table 2. PCR products were sequenced using the Applied Biosystems 3730/3730xl DNA analyzers sequencing system. Allelic profiles and sequence type (ST) were assigned at the PubMLST database (http://pubmlst.org/paeruginosa/).

2.8. Statistical analysis

Between-group comparisons were made using the Fisher's exact test. Statistical significance was defined as a P value < 0.05.

Please cite this article in press as: Takata I, et al., Association of the *exoU* genotype with a multidrug non-susceptible phenotype and mRNA expressions of resistance genes in *Pseudomonas aeruginosa*, J Infect Chemother (2017), http://dx.doi.org/10.1016/j.jiac.2017.08.018