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Original Article

An outbreak of fluoroquinolone-resistant *Pseudomonas aeruginosa* ST357 harboring the *exoU* gene☆

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ABSTRACT

Antimicrobial-resistant isolates of *Pseudomonas aeruginosa* collected from 2005 to 2014 in a university hospital in Kyoto, Japan, were retrospectively analyzed by multilocus sequence typing (MLST), exoenzyme genotype determination, integron characterization, and clinical associations. During the study, 1573 *P. aeruginosa* isolates were detected, and 41 of these were resistant to more than two classes of antimicrobial agents. Twenty-five (61.0%) isolates were collected from urine. All isolates were resistant to ciprofloxacin, 8 (19.5%) isolates showed resistance to imipenem/cilastatin, and 8 (19.5%) isolates showed resistance to meropenem. None of the isolates fulfilled the clinical criteria for multidrug-resistant *P. aeruginosa*. All isolates were negative in the metallo-β lactamase test. Thirty-six (87.8%) isolates were of the *exoS*$^{-}$*exoU*$^{+}$ genotype and 5 (12.2%) isolates were of the *exoS*$^{+}$*exoU*$^{-}$ genotype. Among 36 *exoS*$^{-}$*exoU*$^{+}$ isolates, 33 (80.5%) were ST357, and 3 (7.3%) were ST235. Five isolates of *exoS*$^{+}$*exoU*$^{-}$ were ST186, ST244, ST314, ST508, and ST512. Thirty-three isolates were positive for class 1 integrons and four different class 1 integrons were detected: aminoglycoside (2′) adenyltransferase and chloramphenicol transporter (AadB+CmlA6), OXA-4 β-lactamase and aminoglycoside 3′-adenyltransferase (OXA4+AadA2), AadB alone, and aminoglycoside acetyltransferase alone (AacA31). Among the 41 patients from which the isolates originated, the most common underlying disease was cancer in 16 patients (39%), and 9 patients (22.0%) died during the hospitalization period. There was no statistical correlation between MLST, exoenzyme genotype, and patient mortality. The results indicated outbreaks of fluoroquinolone-resistant *P. aeruginosa* in immunocompromised patients mainly due to the propagation of potentially virulent ST357 isolates possessing the *exoU*$^{+}$ genotype.

1. Introduction

The recent proliferation of multidrug-resistant (MDR) pathogens and the increased number of immunocompromised hosts has created a worldwide healthcare crisis [1]. *Pseudomonas aeruginosa* is one of the common pathogens causing various lethal opportunistic infections, especially in critically-ill patients [2,3]. Conventional antimicrobial agents are not always effective against this organism due to its intrinsically advanced antibiotic resistance mechanisms. In addition, a variety of subpopulations exhibit significant variability in pathogenicity, which is associated with genetic variations in virulence factors that likely affect the prognosis of patients. *P. aeruginosa* possesses one of the largest genomes

Abbreviations: AadA2, aminoglycoside 3′-adenyltransferase; AadB, aminoglycoside (2′) adenyltransferase; AacA31, aminoglycoside acetyltransferase AacA31; CmlA6, chloramphenicol transporter CmlA6; CLSI, Clinical and Laboratory Standards Institute; MBL, metallo-β-lactamase; MDR, multidrug resistant; MIC, minimum inhibitory concentration; MLST, multilocus sequence typing.

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among bacterial pathogens that infect humans with reported sizes of between 5.5 and 7 million base pairs [4]. It has been reported that the *P. aeruginosa* genome is composed of a relatively conserved "core genome" and a highly variable "accessory genome", and the intraspecies diversity is due to the dynamic nature of the accessory genome [5]. One region of the variable "accessory genome" contains integrons associated with the generation of diverse antimicrobial phenotypes. As well as being potential mobile themselves between bacterial species, integrons usually contain several mobile cassette genes that confer resistance to β-lactams or aminoglycosides. Many different types of metallo-β-lactamase (MBL) genes, such as IMP-type and VIM-type, have been found to exist as insertional cassettes in the integrons.

Another key factor associated with the "accessory genome" region of *P. aeruginosa* is the phenotype of type III secretory toxins. In a similar manner to many other pathogenic Gram-negative bacteria, *P. aeruginosa* injects effector proteins into host cells through the type III secretion system [6]. Although four effector proteins, ExoS, ExoT, ExoU, and ExoY, have been found in *P. aeruginosa*, the *exoS*$^+$ and *exoU*$^+$ genotypes are mutually exclusive [7,8] and the *exoU* gene locates in pathogenicity island 2 (PAPI-2) [9]. ExoU, which mediates phospholipase A_2 activity [10,11], has been defined as a major cause of cytotoxicity and epithelial injury and is reported to have a major impact on disease severity [12,13]. The association between fluoroquinolone resistance and the *exoU*$^+$ genotype has been reported as a poor prognostic factor in *P. aeruginosa* infection [14–17]. Therefore, epidemiological determination of the *exoU*$^+$ genotype of isolates is important to predict the outcome of *P. aeruginosa* infections.

In this study, we applied *exoS*/*exoU* genotyping, multilocus sequence typing (MLST), and integron analyses to the in-hospital stock library of fluoroquinolone-resistant *P. aeruginosa* isolates. The results indicated that the outbreak of fluoroquinolone resistance was due to the propagation of potentially virulent ST357 isolates possessing the *exoU*$^+$ genotype. Epidemiological analysis based on the molecular biological classification of *P. aeruginosa* isolates is discussed.

2. Materials and methods

2.1. P. aeruginosa culture collection and susceptibility profile to antibiotics

Clinical isolates of *P. aeruginosa* were collected prospectively from 2005 to 2014 in the university hospital at Kyoto Prefectural University of Medicine. During the study period, 1573 *P. aeruginosa* isolates were detected. As an antimicrobial susceptibility test, minimum inhibitory concentration (MIC) breakpoints and quality-control protocols were used according to standards established by the Clinical and Laboratory Standards Institute (CLSI). Antimicrobial breakpoints were based on CLSI M100-S25 [18]. Forty-one isolates were defined as being non-susceptible to at least one agent in ≥3 antimicrobial categories according to the publication by Magiorakos and colleagues [19]. All isolates were resistant to fluoroquinolone. The isolates were cultured in tryptic soy broth under routine laboratory conditions (37 °C, 200 rpm, 24 h), and chromosomal DNA was extracted from the isolates using a QIA amp DNA Mini Kit (#51304, Qiagen, Hilden, Germany). The sodium mercaptopropionic acid (SMA)-used double-disk synergy test (DDST) was conducted for the detection of MBL-producing strains, according to the recommendations of the manufacturer (Eiken Kagaku, Tochigi, Japan) [20].

2.2. Exoenzyme genotyping

Diagnostic polymerase chain reaction (PCR) primer sets for genotyping type III secretory toxin genes *exoS* and *exoU* were prepared for this study. Briefly, six sets of PCR primers were designed from the consensus regions in the *exoS* and *exoU* genes among the clinical isolates of *P. aeruginosa* (Table 1). By using genomic DNA purified from cultures of isolates as PCR templates, PCR amplification was performed under the following PCR conditions: denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s for 28 cycles. The genotypes were determined by 2% agarose gel electrophoresis [21].

2.3. Multilocus sequence typing (MLST)

MLST, which is based on sequence analysis of the internal portions of seven housekeeping genes, was performed as described previously with some modifications [22]. The following seven genes, *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*, were amplified by PCR using the primer pairs described in Table 2. PCR conditions were as follows: denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s for 30 cycles. Amplification products were purified using the PCR Clean-Up kit (#28104, Qiagen), and sequencing of the PCR amplicons was performed by a DNA sequencing service (Eurofinsgenomics Inc., Tokyo, Japan) using the seven primer sets (Table 2). The DNA sequences of each gene were compared with sequences in the MLST database (https://pubmlst.org/paeruginosa/) for the assignment of allelic numbers and sequence types [23].

2.4. Detection of integrons and gene cassettes

The presence of integron genes was assessed by PCR amplification of an internal fragment of the integrase genes (*intI1*, *intI2*, and *intI3*) using PCR primer sets described previously [24,25] (Table 2). The variable gene cassette regions within class 1 integrons were amplified using the primer pairs CS5 and CS3 (Table 2). After agarose gel electrophoresis and band excision, the PCR products were purified using a PCR Clean-Up kit (Qiagen). The DNA sequences were determined by a DNA sequencing service (Eurofinsgenomics Inc.) and analyzed using the basic local alignment search tool program (BLAST, https://blast.ncbi.nlm.nih.gov/, National Center for Biotechnology Information, Bethesda, MD, USA) for gene cassette screening.

2.5. eBURST groups and clonal complexes

eBURST group analysis, which displays the most likely patterns of evolutionary descent within each clonal complex, was performed using eBURST version 3 [26,27].

2.6. Collection of clinical data

The medical records of patients infected with, or colonized by, drug-resistant *P. aeruginosa* were reviewed retrospectively.

2.7. Statistical analysis

Statistical calculations were made using the InStat version 4.0 software (GraphPad Software Inc., La Jolla, CA, USA). *P* values were calculated using the two-tailed Fisher's exact test for categorical variables, and a P value < 0.05 was considered a statistically significant difference.

2.8. Nucleotide sequence accession number

The sequence data of OXA-4 β-lactamase and aminoglycoside 3′-adenyltransferase (OXA-4 + AadA2) and aminoglycoside (2′) adenyltransferase and the chloramphenicol transporter (AadB + CmlA6) from this study were deposited into the DDBJ/EMBL/GenBank

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