Mechanisms of resistance to fluoroquinolones and carbapenems in Pseudomonas putida

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Objectives: Pseudomonas putida is an uncommon opportunistic pathogen, usually susceptible to antimicrobial agents. Data concerning resistance to antimicrobial agents in clinical P. putida isolates are limited.

Patients and methods: Susceptibilities to fluoroquinolones, carbapenems and other antibiotics were characterized in five clinical isolates of P. putida recovered from different patients with urinary tract infections as causative pathogens. Fluoroquinolone and carbapenem resistance were characterized genetically by the methods of PCR and DNA sequencing. Outer membrane protein (OMP) profiles were characterized by SDS–PAGE.

Results: Four of five isolates were resistant or intermediate to both fluoroquinolones and carbapenems. Nucleotide sequences in the quinolone resistance-determining regions suggested that amino acid mutations such as Thr-83 → Ile in GyrA and Glu-469 → Asp in GyrB may contribute to high resistance to fluoroquinolones. Four metallo-β-lactamase-producing isolates that showed resistance to carbapenems carried the IMP-type metallo-β-lactamase genes. A combined effect of reduced production of 46 kDa OMP and metallo-β-lactamase production was shown by a P. putida isolate exhibiting the highest MICs of carbapenems.

Conclusions: This study identified mechanisms of resistance to fluoroquinolones and carbapenems in clinical P. putida isolates.

Keywords: Pseudomonas spp., antibiotic resistance, outer membrane protein, OMP, β-lactamases

Introduction

Pseudomonas putida, a non-fermenting Gram-negative bacillus, is an opportunistic human pathogen responsible for bacteremia and sepsis in neonatal, neutropenic and cancer patients, as well as urinary tract infections (UTIs).1–4 Although uncommon, P. putida can be a cause of nosocomial infections in compromised hosts.5

Most P. putida are susceptible to antimicrobial agents such as carbapenems, fluoroquinolones and aminoglycosides.5–7 However, clinical isolates of P. putida that produce metallo-β-lactamases conferring resistance to β-lactams including carbapenems, have been reported.3,8,9 Further, metallo-β-lactamase-producing isolates that showed resistance to ciprofloxacin, gentamicin and tobramycin in addition to β-lactams were recently reported.3 Emergence of multiple-drug-resistant P. putida has become a cause of difficulty in treating infections and poses a risk of nosocomial transmission.

In a few previous studies, antibiotic resistance in P. putida has been characterized with respect to production of metallo-β-lactamases and characteristics of efflux systems.3,4,8–12 Carbapenem-resistant P. putida frequently produce IMP- and VIM-type metallo-β-lactamases according to reports from Europe, Korea and Japan.3,8,9,9a Efflux systems such as TtgABC, MepABC, TtgDEF and ArpABC can also contribute to multiple-drug resistance in P. putida.10–12 Pseudomonas aeruginosa has the capacity to rapidly become resistant during the course of treatment.13

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although whether P. putida has the same potential is unclear. To assess risks that antibiotic resistance will emerge, abundant data concerning resistance to antimicrobial agents in clinical P. putida isolates are required. In this study, we determined susceptibilities to antimicrobial agents including fluoroquinolones and carbapenems and characterized mechanisms of resistance to fluoroquinolones and carbapenems, in clinical P. putida isolates recovered as pathogens causing UTIs during a 1 year period at our hospital.

Patients and methods

Patients

Five patients (four males and one female) diagnosed with acute, repeated or chronic UTIs caused by P. putida at Hamamatsu University Hospital were included in our study from October 2001 through September 2002.

Bacterial strains and microbiological methods

Strains used in this study were five clinical isolates of P. putida recovered from different patients as causative pathogens. Identification was performed by standard biochemical tests in our clinical microbiology laboratory. All isolates were obtained from urine. The SpeI-restricted fragment patterns of the chromosomal DNA of these five isolates varied (Figure 1). Bacteria were stored at –70°C in heart infusion broth (Nissui Pharmaceutical, Tokyo, Japan) containing 20% glycerol. Subsequently, bacteria were inoculated on heart infusion agar plates (Nissui Pharmaceutical) and incubated at 37°C overnight. MICs were determined by an agar dilution method as described by the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS).14

Susceptibility testing was performed using Mueller–Hinton agar (Nippon Becton Dickinson, Tokyo, Japan) in accordance with the manufacturer’s instructions. MIC interpretative criteria for ceftazidime, imipenem, meropenem, norfloxacin, levofloxacin, gatifloxacin, gentamicin, amikacin and minocycline followed those of the CLSI/NCCLS.14 MIC breakpoints of other antimicrobial agents were not defined.

Antimicrobial agents

Antibiotics used included amikacin, ampicillin, biapenem and kanamycin (Meiji Seika Kaisha, Tokyo, Japan), cefaloridine (Shionogi Pharmaceutical, Osaka, Japan), ceftazidime (GlaxoSmithKline, Tokyo, Japan), gentamicin (Schering-Plough, Tokyo, Japan), imipenem (Banyu Pharmaceutical, Tokyo, Japan), panipenem (Sankyo, Tokyo, Japan), meropenem (Sumitomo Pharmaceutical, Osaka, Japan), norfloxacin and gatifloxacin (Kyorin Pharmaceutical, Tokyo, Japan), levofloxacin and sitafloxacin (Daichi Pharmaceutical, Tokyo, Japan), pazufloxacin (Dainippon Pharmaceuticals, Tokyo, Japan), pazufloxacin (Taishotoyama Pharmaceutical, Tokyo, Japan) and minocycline (Wyeth, Tokyo, Japan).

Amplification and DNA sequencing of quinolone resistance-determining regions

Chromosomal DNA was extracted from P. putida isolates as previously described.15 PCR amplification was performed with specific primer sets. A primer set of 5’-gaacgctgagcatagtgcgtgcag-3’ and 5’-gccaagggtaaccgctgag-3’ amplified a 417 bp fragment of the quinolone resistance-determining regions (QRDRs) of the gyrA gene from positions 115 to 531.16 A primer set of 5’-agcagcggcggagct-3’ and 5’-tacagcggcggagct-3’ amplified a 739 bp fragment of the QRDRs of the gyrB gene from positions 1073 to 1811.17 A primer set of 5’-tctagcggcggatggtggg-3’ and 5’-agcagcggcggagct-3’ amplified a 262 bp fragment of the QRDRs of the parC gene from positions 158 to 419.18 Amplifications were carried out with Advantage-GC2 enzyme (BD Biosciences Clontech Japan, Tokyo, Japan) according to the manufacturer’s instructions. The QRDRs were sequenced using a BigDye terminator v3.0 Taq cycle sequencing ready reaction kit with AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA, USA) and an automated DNA sequencing system (ABI PRISM 310 genetic analyzer, Applied Biosystems, Foster City, CA, USA).

Genetic detection of metallo-ß-lactamase genes

Detection of the IMP- and VIM-type metallo-ß-lactamase genes was carried out by PCR amplification of chromosomal DNA with the following sets of specific primers: for the IMP-type gene, IMP-1A (5’-ctacgccgacagcttgcgtgcag-3’) and IMP-1B (5’-gaacagcatgtttggctgag-3’); for the VIM-1-type gene, VIM-1A (5’-tctacgccgacagcttgcgtgcag-3’) and VIM-1B (5’-gcttcctgacacgcttgcgtgcag-3’); and for the VIM-2-type gene, VIM-2A (5’-atgcttcctgacacgcttgcgtgcag-3’) and VIM-2B (5’-ctacgccgacagcttgcgtgcag-3’).19 Amplifications were carried out using Ex Taq enzyme (Takara Bio, Ohtsu, Japan) according to the manufacturer’s recommendations.

Preparation of outer membrane proteins

Outer membrane proteins (OMPs) were prepared as previously described.25 Samples were analysed by SDS–PAGE.
Fluoroquinolone/carbapenem resistance in *P. putida*

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mutations</th>
<th>MIC (mg/L)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>GyrA</td>
<td>GyrB</td>
</tr>
<tr>
<td><em>P. putida</em> HU2001-412</td>
<td>Thr-83→Ile, Ser-136→Ala</td>
<td>Glu-469→Asp</td>
</tr>
<tr>
<td><em>P. putida</em> HU2001-419</td>
<td>Thr-83→Ile, Ser-136→Ala</td>
<td>Glu-469→Asp</td>
</tr>
<tr>
<td><em>P. putida</em> HU2002-467</td>
<td>Thr-83→Ile, Ser-136→Ala</td>
<td>Glu-469→Asp</td>
</tr>
<tr>
<td><em>P. putida</em> HU2001-451</td>
<td>Thr-83→Ile</td>
<td>Arg-442→Lys, Asp-489→Glu</td>
</tr>
<tr>
<td><em>P. putida</em> HU2001-429</td>
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NOR, norfloxacin; LVX, levofloxacin; SPX, sparfloxacin; GAT, gatifloxacin; PAZ, pazufloxacin; SIT, sitafloxacin.

*ª* Amino acid mutations were identified compared with the sequences of a fluoroquinolone-susceptible strain, *P. putida* HU2001-429.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>β-Lactamase gene</th>
<th>MIC (mg/L)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>bblaimp-1</td>
<td>blavim-1</td>
</tr>
<tr>
<td><em>P. putida</em> HU2001-412</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>P. putida</em> HU2001-419</td>
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</tr>
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</tr>
<tr>
<td><em>P. putida</em> HU2001-429</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

ªOMP, outer membrane protein. Production of 46 kDa protein was undetectable (U) or detectable (D) by SDS–PAGE.

**Results**

**Susceptibility**

Results of susceptibility testing for fluoroquinolones and carbapenems are summarized in Tables 1 and 2, respectively. MICs of β-lactams ranged from >128 mg/L for ampicillin and cefaloridine, 2 to >128 mg/L for cefazidime, 1 to 128 mg/L for imipenem, 0.5 to >128 mg/L for panipenem, 4 to >128 mg/L for meropenem and 1 to >128 mg/L for biapenem. MIC ranges of aminoglycosides and minocycline were as follows: 0.25 to 0.125 and 8 mg/L. In the five isolates, the MIC range for minocycline was between ≤0.125 and 8 mg/L.

**Fluoroquinolone resistance**

We determined the nucleotide sequences in the QRDRs of the *gyrA*, *gyrB* and *parC* genes in the five isolates of *P. putida*. Amino acid mutations in four fluoroquinolone-resistant isolates, HU2001-412, HU2001-419, HU2001-451 and HU2002-467, were identified by comparison with the sequences of the susceptible isolate, HU2001-429 (Table 1). Fluoroquinolone-resistant isolates had additional mutations such as Thr-83→Ile and Ser-136→Ala in GyrA; Glu-469→Asp, Arg-442→Lys and Asp-489→Glu in GyrB; and Thr-105→Pro, Val-124→Ala and Ser-136→Ala in ParC. Homologies between fluoroquinolone-resistant isolates, HU2001-412, HU2001-419, HU2001-451 and HU2002-467, and susceptible isolate HU2001-429 in the QRDR of the *gyrA* gene were 96.8% (98.3%), 96.8% (98.3%), 93.9% (97.4%) and 96.8% (98.3%) at the nucleotide (amino acid) level, respectively. Those in the *gyrB* gene were 89.5% (96.3%), 88.9% (96.3%), 99.4% (98.1%) and 96.8% (98.3%) at the nucleotide (amino acid) level, respectively. Those in the *parC* gene were 89.5% (96.3%), 88.9% (96.3%), 93.9% (97.4%) and 96.8% (98.3%) at the nucleotide (amino acid) level, respectively.

**Carbapenem resistance**

Of five *P. putida* isolates, four showing carbapenem resistance carried the IMP-type metallo-β-lactamase gene, whereas the VIM-type metallo-β-lactamase genes were not detected by PCR (Table 2). MIC ranges of carbapenems in *P. putida* carrying the IMP-type metallo-β-lactamase genes were as follows: 8 to 128 mg/L for imipenem, 32 to >128 mg/L for panipenem,
128 mg/L or greater for meropenem and 32 to >128 mg/L for biapenem. In *P. putida* HU2001-451, which showed the highest MICs of carbapenems among the five isolates, production of 46 kDa OMP was undetectable by SDS–PAGE, whereas those of *P. putida* HU2001-412, HU2001-419, HU2001-429 and HU2002-467 were detected similarly (Table 2).

**Discussion**

In this study, we characterized susceptibilities to fluoroquinolones and carbapenems in five clinical isolates of *P. putida* isolated from different patients with acute, repeated or chronic UTIs. All five isolates showed different PFGE genotypes, which suggested that none of the infections caused by these *P. putida* were nosocomial. Four of five isolates were resistant or intermediate to both fluoroquinolones and carbapenems. Three isolates showed high resistance (>128 mg/L) to all fluoroquinolones examined except for sitafloxacin. Among the fluoroquinolones investigated in this study, sitafloxacin showed superior potency against the *P. putida* isolates. These isolates highly resistant to fluoroquinolones were also resistant to carbapenems and minocycline. All isolates were susceptible to aminoglycosides such as amikacin.

Studies of fluoroquinolone resistance in *P. putida* are limited. In *P. aeruginosa*, major mechanisms responsible for fluoroquinolone resistance include amino acid mutations in DNA gyrase or topoisomerase IV caused by mutations in the QRDRs of GyrA and ParC, whereas some reports have suggested involvement of mutations of GyrB in fluoroquinolone resistance. A secondary resistance mechanism in *P. aeruginosa* that involves efflux systems contributes to reduced susceptibility to fluoroquinolones. In this study, amino acid alterations in the QRDRs of GyrA, GyrB and ParC were compared between five clinical isolates of *P. putida*. Fluoroquinolone-resistant *P. putida* had additional mutations such as Thr-83→Ile in GyrA and Glu-469→Asp in GyrB, which corresponded to mutations found in fluoroquinolone-resistant *P. aeruginosa*. These results indicate that amino acid mutations in the QRDRs such as Thr-83→Ile in GyrA and Glu-469→Asp in GyrB may contribute to high resistance in fluoroquinolones, although when transformation by plasmids carrying the wild-type gyrA, gyrB or parC genes into such isolates would lower the MICs of fluoroquinolones was not determined. The MIC range of sitafloxacin was between $\leq 0.125$ and 8 mg/L for the five *P. putida* isolates. Although previous reports have shown that overexpression of the TtgABC, MepABC, TtgDEF and Arp-ABC efflux systems can also contribute to multiple-drug resistance in *P. putida*, the role of efflux systems remained unclear in this study.

Carbapenem resistance in *P. putida* caused by production of metallo-β-lactamases has been reported. These metallo-β-lactamases found in *P. putida* included IMP- and VIM-types. In the four carbapenem-resistant *P. putida*, production of metallo-β-lactamases was detected by a disc diffusion method (data not shown). These isolates carried the IMP-type metallo-β-lactamase genes, whereas the VIM-type metallo-β-lactamase genes were not detected by PCR. Prevalence of metallo-β-lactamase-producing *P. putida* is an important clinical problem, representing a reservoir of genetic determinants of β-lactam resistance. In *P. aeruginosa*, other major mechanisms of carbapenem resistance include mutational impermeability arising via loss of OprD—a porin-forming transmembrane channel accessible to carbapenems but not other β-lactams—apart from production of metallo-β-lactamases. Loss of OprD results in resistance to imipenem and reduced susceptibility to meropenem in *P. aeruginosa*. In *P. putida* HU2001-451, showing the highest MICs (≥2128 mg/L) of all carbapenems among four carbapenem-resistant isolates, production of 46 kDa OMP was reduced in comparison with those of other isolates. The OMP profiles of *P. putida* HU2001-451 were similar to those of carbapenem-resistant *P. aeruginosa*, in which reduced production of OprD was identified in our previous study. These results showed a combined effect of reduced production of 46 kDa OMP coexisting with production of metallo-β-lactamases on carbapenem resistance in the isolate, although whether other β-lactamases had relevance to carbapenem resistance was unclear. In conclusion, we characterized fluoroquinolone and carbapenem resistance in clinical isolates of *P. putida*. Our results indicate that amino acid mutations in the QRDRs, such as Thr-83→Ile in GyrA and Glu-469→Asp in GyrB, can contribute to high resistance to fluoroquinolones in *P. putida*. Four metallo-β-lactamase-producing *P. putida* isolates that showed resistance to carbapenems carried the IMP-type metallo-β-lactamase gene. We found a combined effect of reduced production of 46 kDa OMP and production of metallo-β-lactamases that enhanced carbapenem resistance in an isolate of *P. putida* showing the highest MICs of carbapenems.

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Fluoroquinolone/carbapenem resistance in \textit{P. putida}


19. Deleted.


