Variations in Ceftazidime and Amoxicillin-Clavulanate Susceptibilities within a Clonal Infection of *Burkholderia pseudomallei*†

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A patient with a clonal infection of *Burkholderia pseudomallei* had subpopulations with ceftazidime and amoxicillin-clavulanate susceptibilities that differed among the clinical specimens. Resistance was associated with a novel Cys69Tyr substitution in the Ambler class A β-lactamase. Susceptibility testing of multiple colony variants from different sites should be performed for patients with culture-confirmed melioidosis.

*Burkholderia pseudomallei*, an environmental bacterium found throughout Southeast Asia and northern Australia, causes melioidosis. Recommended treatment regimens consist of administration of an initial intravenous agent (ceftazidime, imipenem, or amoxicillin-clavulanate) followed by prolonged oral therapy (3). Despite such therapy, recurrent disease occurs in up to 16% of patients; up to 75% of the recurrences are relapses caused by the same strain, while the remainder are new infections by different strains (15, 22). In most cases, even those with an apparently mixed population of colony variants, infection is due to a single strain rather than multiple strains (14, 18).

Recurrences are occasionally caused by isolates with acquired antibiotic resistance (4, 6), but detailed analysis of such cases is lacking. In two studies, neither of which determined mechanisms of resistance, randomly amplified polymorphic DNA profiles of resistant isolates from recurrences of disease were the same as those determined for the original strain in some cases (11), while band changes were present in other cases (6). Tribuddharat et al. showed that acquired resistance to ceftazidime and amoxicillin-clavulanate was associated with single mutations in the Ambler class A β-lactamase (21) but did not perform typing of the resistant and susceptible isolates. We noted a patient with multiple *B. pseudomallei* isolates of various levels of ceftazidime and amoxicillin-clavulanate susceptibilities during a single infection. In this retrospective study, we typed these isolates and determined the molecular basis of β-lactamase-associated resistance.

A 57-year-old diabetic male presented with severe pneumonia, which was unresponsive to ampicillin-sulbactam. *B. pseudomallei* was isolated from three specimens, at days 1, 9, and 17 of admission, during which he was treated with ceftazidime. The three stocked isolates were subcultured, and three to five single colonies from each subculture were picked for pulsed-field gel electrophoresis (PFGE) (19), using SpeI and XbaI and a Chef DR-II system (Bio-Rad, Hercules, CA). Restriction patterns were compared using the Dice coefficient (*F* value), and cluster analysis was performed by the use of the unweighted-pair group method using average linkages and Bionumerics software, version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium). Etests were used to measure MICs (AB Biodisk, Solna, Sweden), with *B. pseudomallei* NCTC 13178 and *Pseudomonas aeruginosa* ATCC 27853 used as controls. PCR amplification and sequencing of the complete class A (*penA*) and class D (*oxa*) β-lactamase genes were carried out (9, 17). Sequences were then compared to those of *B. pseudomallei* strain K96243 (10).

A total of five different isolates from the three specimens were studied (Table 1). These showed two patterns of antibiotic susceptibility: intermediate susceptibility to ceftazidime and amoxicillin-clavulanate in the first pattern and ceftazidime resistance and amoxicillin-clavulanate susceptibility in the second pattern. All isolates were susceptible to imipenem, meropenem, chloramphenicol, and trimethoprim-sulfamethoxazole. There was a heterogenous population within the first stocked isolate, with ceftazidime-resistant white and yellow colonies as well as colonies that were intermittently susceptible. The resistant subpopulations had not been noted during primary processing of the specimens. Subsequent isolates, from sputum specimens, showed intermediate ceftazidime susceptibility, followed by ceftazidime resistance. PFGE using XbaI showed indistinguishable profiles for all patient isolates tested (data not shown). With SpeI, all showed the same 18-band profile, except for the 76161y isolate, which had one absent band between 216.9 and 244.4 kbp (*F = 0.97*) (Fig. 1). Thus, all isolates from this patient appear to represent the same strain (or a strain very closely related, in the case of 76161y), despite differences in antibiotic susceptibility and color.

All the isolates, regardless of the antibiotic susceptibility, had a silent mutation at position 750 of the *oxa* gene, resulting in the same class D β-lactamase amino acid sequence as that seen with strain K96243. Cefazidime and amoxicillin-clavulanate resistance do not appear to be mediated by the class D β-lactamase (12, 17).

All three ceftazidime-resistant isolates in this study had a single nucleotide change in *penA*, leading to a Cys69Tyr substitution, according to the Ambler numbering scheme (1). Situated next to the critical catalytic serine residue 70, residue 69 appears important in determining substrate specificity in
SHV-1, another class A β-lactamase (8). Substitutions with Tyr, Phe, and Lys increased the hydrolysis of ceftazidime, while substitutions with Ile, Leu, and Val resulted in β-lactamase inhibitor resistance (8). To date, the only reported mutation associated with ceftazidime resistance in *B. pseudomallei* is Pro167Ser in the class A β-lactamase, which is also located within a conserved motif of the catalytic site (9, 21). The mutation described in the present study appears to be novel in *B. pseudomallei*. Unchanged *penA* sequences were seen in all the ceftazidime-intermediate isolates in this study, and in a ceftazidime-resistant strain in another study (16), suggesting the presence of other unidentified mechanisms of reduced susceptibility to ceftazidime. These include putative efflux pumps (13) and at least five other β-lactamases (10) which are present in the *B. pseudomallei* genome but have not yet been conclusively associated with β-lactam resistance.

The stock isolate from the first specimen contained ceftazidime-intermediate and ceftazidime-resistant subpopulations, with the latter not being detected initially. We cannot exclude the possibility that the 76161w and 76161y colonies represent laboratory mutants or contaminants. However, ceftazidime resistance is rare in *B. pseudomallei*, having been reported to be present in just 0.5 to 0.7% of strains (5, 20). Of 181 other strains tested in our laboratory with a MIC<sub>50</sub> of 2 μg/ml and MIC<sub>90</sub> of 3 μg/ml, none were resistant to ceftazidime (I. C. Sam, K. H. See, and S. D. Puthucheary, unpublished data). It is unlikely that ceftazidime resistance would arise within the ceftazidime-intermediate subpopulation in the absence of antibiotic pressure in the laboratory. Another possibility is contamination with ceftazidime-resistant isolate 3192; however, no yellow colonies were seen upon subculturing of isolate 3192. An alternative explanation is that resistant colonies 76161w and 76161y were present in low numbers in the initial blood culture and were undetected. *B. pseudomallei* appears to exhibit multiple phenotypes within a clonal population and can switch between them in response to the environment, resulting in variations in morphological appearance, intracellular replication, and persistence (2). Highly resistant small-colony variants of *B. pseudomallei* can revert to the parental (larger) size phenotype and antibiotic susceptibility (7). The clinical relevance of phenotypic switching is not known, but it may contribute to the propensity of *B. pseudomallei* infections to reoccur despite treatment.

For this case, we show that a single infectious clonal population of *B. pseudomallei* may contain subpopulations with differing ceftazidime and amoxicillin-clavulanate susceptibilities, that these susceptibilities are associated with a single nucleotide substitution in the *penA* gene, and that the relative proportions of these subpopulations may differ among specimens over time. Earlier treatment with ampicillin-sulbactam may have contributed to the initial development of reduced susceptibility to β-lactams; subsequent ceftazidime treatment ultimately led to selection of the ceftazidime-resistant subpopulation. Therefore, it is important for patients under treatment to have a detailed microbiological follow-up, including susceptibility testing of multiple colony variants from different sites.

**Nucleotide sequence accession numbers.** The *penA* sequences for isolates 76161 and 3192 and the *oxa-1* sequence for isolate 76161, representing the three different sequences seen in this case, were deposited in GenBank under accession numbers FJ147201 to FJ147203.

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**TABLE 1. Details of the *B. pseudomallei* isolates analyzed**

<table>
<thead>
<tr>
<th>Specimen no. (type)</th>
<th>Date of specimen (days after admission)</th>
<th>Isolate</th>
<th>Phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ceftazidime MIC (μg/ml)</th>
<th>Amoxicillin-clavulanate MIC (μg/ml)</th>
<th>Class A β-lactamase protein sequence change&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Class D β-lactamase protein sequence change&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (blood)</td>
<td>1</td>
<td>76161</td>
<td>CAZ-I, AMC-I</td>
<td>24</td>
<td>16</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>1 (blood)</td>
<td>1</td>
<td>76161y</td>
<td>Yellow, CAZ-R, AMC-S</td>
<td>256</td>
<td>2</td>
<td>Cys69Tyr</td>
<td>No change</td>
</tr>
<tr>
<td>1 (blood)</td>
<td>1</td>
<td>76161w</td>
<td>White, CAZ-R, AMC-S</td>
<td>256</td>
<td>2</td>
<td>Cys69Tyr</td>
<td>No change</td>
</tr>
<tr>
<td>2 (sputum)</td>
<td>9</td>
<td>0907</td>
<td>CAZ-I, AMC-I</td>
<td>24</td>
<td>16</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>3 (sputum)</td>
<td>17</td>
<td>3192</td>
<td>CAZ-R, AMC-S</td>
<td>256</td>
<td>2</td>
<td>Cys69Tyr</td>
<td>No change</td>
</tr>
</tbody>
</table>

<sup>a</sup> CAZ, ceftazidime; AMC, amoxicillin-clavulanate; I, intermediate susceptibility; R, resistant; S, susceptible.

<sup>b</sup> Compared to *B. pseudomallei* strain K96243 (10).

<sup>c</sup> All isolates had a silent mutation of C to A at nucleotide position 750.

**FIG. 1.** PFGE of SpeI-digested DNA of isolates obtained from the patient’s three specimens, showing indistinguishable profiles, except for a 1-band difference in isolate 76161y (lane 16). Lanes 1, 7, 14, and 19: *Salmonella enterica* serotype Braenderup H9812; lane 2, isolate 76161; lane 3, isolate 0907; lane 4, isolate 3192; lane 5, *B. pseudomallei* NCTC 13178; lane 6, *B. pseudomallei* ATCC 23343; lanes 8 to 13 and lanes 17 and 18, other colonies randomly picked from the three specimens; lane 15, isolate 76161w; lane 16, isolate 76161y; lane 20, *B. pseudomallei* clinical strain 7722 from a different patient.
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