Whole-Genome Sequencing Identifies Emergence of a Quinolone Resistance Mutation in a Case of Stenotrophomonas maltophilia Bacteremia

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Whole-genome sequences for Stenotrophomonas maltophilia serial isolates from a bacteremic patient before and after development of levofloxacin resistance were assembled de novo and differed by one single-nucleotide variant in smeT, a repressor for multidrug efflux operon smeDEF. Along with sequenced isolates from five contemporaneous cases, they displayed considerable diversity compared against all published complete genomes. Whole-genome sequencing and complete assembly can conclusively identify resistance mechanisms emerging in S. maltophilia strains during clinical therapy.

Stenotrophomonas maltophilia is an aerobic, nonfermenting, and motile Gram-negative bacterium that is increasingly recognized as a cause of hospital-acquired infections, with crude mortality rates of 14 to 69% in cases of bacteremia (1). Treatment of S. maltophilia infections is challenging due to the pathogen’s intrinsic resistance to many antibiotic classes via drug efflux pumps, beta-lactamase production, and decreased membrane permeability (1). Resistance phenotypes are known to change during the course of treatment, which complicates interpretation of automated drug susceptibility testing (DST) results (2). A mutant strain of S. maltophilia with emerging resistance to tetracycline, chloramphenicol, and quinolones was previously characterized following in vitro tetracycline selection (3, 4). However, little is known about the genetic and molecular mechanisms underlying acquired resistance in the clinical setting—particularly for quinolones, as, in contrast to other Gram-negative organisms, the quinolone-resistance determining region (QRDR) of topoisomerase genes is often unaltered (5). In this report, we describe the first reported use of whole-genome sequencing (WGS) in serial clinical isolates to definitively identify an acquired quinolone resistance mutation in S. maltophilia.

WGS was performed for the initial and subsequent S. maltophilia blood culture isolates from a patient for whom acquired quinolone resistance was observed (patient 1) and five other patients (patients 2 to 6) selected from a 2-month period in 2013 at The Mount Sinai Hospital. Patient 1 was a 56-year-old man requiring placement of an intrahepatic shunt for complications of a Whipple procedure, which was followed by several episodes of bacteremia that were treated with multiple courses of antimicrobials, including levofloxacin. Two months later, another bacteremia developed, and blood cultures intermittently grew S. maltophilia despite antimicrobial therapy. Automated DST by Vitek2 (bioMérieux, Marcy l’Étoile, France) showed that the first S. maltophilia isolate acquired (ISMMS2) was susceptible to fluoroquinolones and trimethoprim-sulfamethoxazole (SXT). After treatment with ciprofloxacin the bacteremia initially cleared, but blood cultures 9 days later again grew S. maltophilia (isolated as ISMMS2R), now resistant to fluoroquinolones while still susceptible to SXT. Ciprofloxacin was stopped and intravenous SXT was given; subsequent cultures did not grow S. maltophilia.

Standard culturing and susceptibility testing for levofloxacin and SXT were performed by automated broth microdilution with Vitek2. Antimicrobial sensitivities were reported and interpreted according to the 2015 CLSI guidelines for S. maltophilia (6). Isolates were then stock and frozen at −80°C. Levofloxacin and SXT susceptibilities for all isolates in this study were later confirmed by Etest (bioMérieux) at 24 h. To prepare for sequencing, isolates were grown from single colonies in tryptic soy broth, and DNA extraction was performed as previously described (7).

Sequencing was performed on the PacBio RSII platform (Pacific Biosciences, Menlo Park, CA), and reads were assembled de novo using PacBio’s Hierarchical Genome Assembly Process (version 3) (8). Pairwise comparison and variant calling were performed with MUMmer 3.23 (9). Mugsy 2.2 (10) was used to align whole-genome sequences for phylogenetic reconstruction with RAxML-8.0.2 (11), and rearrangements were visualized with Mauve 2.4.0 (12). Additional details, including Sanger validation methods and sequence accession numbers, are in the supplemental methods.

Two complete whole-genome sequences were derived from patient 1’s isolates before and after the change in levofloxacin MIC sequences for Stenotrophomonas maltophilia serial isolates from a bacteremic patient before and after development of levofloxacin resistance were assembled de novo and differed by one single-nucleotide variant in smeT, a repressor for multidrug efflux operon smeDEF. Along with sequenced isolates from five contemporaneous cases, they displayed considerable diversity compared against all published complete genomes. Whole-genome sequencing and complete assembly can conclusively identify resistance mechanisms emerging in S. maltophilia strains during clinical therapy.

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and compared to whole-genome sequences of five control \textit{S. maltophilia} isolates (patients 2 to 6). All sequences were de novo assembled, i.e., without regard to reference assemblies. Table 1 summarizes the relative dates of collection, antimicrobial susceptibility results, and assembly statistics.

Assembled genome sequences for patient 1’s isolates before (ISMMS2) and after (ISMMS2R) observation of levofloxacin resistance were compared directly and were identical except for one single-nucleotide variant (SNV) and five one-base indels. Sanger sequencing confirmed the presence of the SNV but identified the indels as homopolymer assembly errors. Coding domain sequence predictions for the surrounding locus (Fig. 1A) revealed that the SNV was inside \textit{smeT}, which creates a premature stop codon that disrupts function (Fig. 1A and B).

Figure 1B shows an amino acid sequence alignment comparing \textit{smeT} in D457 and D457R to aligned sequences from our seven \textit{S. maltophilia} genomes in GenBank. The distances of tens of thousands of SNVs seen in our phylogeny suggest that the natural diversity of pathogenic \textit{S. maltophilia} is greater than that captured by the current set of reference assemblies, even within a single hospital setting.

Recombination is not an obvious source of diversity in our \textit{S. maltophilia} isolates. Figure S3 in the supplemental material depicts whole-genome alignments between the four clinical isolates where assembly produced a circularized chromosome and the four GenBank references, showing small areas of nonhomology separating large regions of significant homology occurring generally in the same order for each genome. ISMMS2 and ISMMS2R are structurally identical, as expected for serial isolates, while recombination events among other strains are limited to small, 1- to 2-kb, segments. Epigenetics motif analysis also suggests that the isolates are not related. Table S1 in the supplemental material shows different motifs in isolates from separate patients, implicating differences in type II and III restriction modification systems between the isolates more likely to be caused by interstrain or interspecies horizontal transfer of methyltransferases than by in-strain mutations (16). Together, these observations demonstrate that transmission did not occur among these six cases and that whole-genome sequencing can comprehensively capture genetic distances and structural variants among diverse clinical isolates of \textit{S. maltophilia}.

This is the first report of WGS on serial isolates to characterize the emergence of a resistance mutation in \textit{S. maltophilia} during antibiotic treatment of an active infection. In contrast to studies sequencing highly resistant strains of \textit{S. maltophilia} to reveal various intrinsic and acquired antibiotic resistance genes (17, 18), where it remains difficult to assess their relative importance to the phenotype, performing WGS on serial isolates as resistance emerges \textit{in vivo} allows the causative mutation(s) to be captured. In our patient, the mutation was a SNV that replicates a variant ob-

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time of collection$^b$ (days)</th>
<th>Isolate name</th>
<th>Susceptibility (MIC [mg/liter])</th>
<th>Assembly quality</th>
<th>Depth of coverage</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Levo</td>
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<td></td>
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<td></td>
<td>Vitek2</td>
<td>Etest</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>ISMMS2</td>
<td>S (0.5)$^c$</td>
<td>S (1)</td>
<td>S (&lt;20)</td>
</tr>
<tr>
<td>1</td>
<td>+10</td>
<td>ISMMS2R</td>
<td>R (&gt;32)$^c$</td>
<td>R (16)</td>
<td>S (1)</td>
</tr>
<tr>
<td>2</td>
<td>−26</td>
<td>ISMMS3</td>
<td>S (0.25)</td>
<td>S (0.38)</td>
<td>U (60, &lt;20)$^d$</td>
</tr>
<tr>
<td>3</td>
<td>+14</td>
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<tr>
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<td>ISMMS5</td>
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<tr>
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<td>0</td>
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<tr>
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<td>+2</td>
<td>ISMMS7</td>
<td>S (1)</td>
<td>S (0.75)</td>
<td>S (&lt;20)</td>
</tr>
</tbody>
</table>

$^a$ Levo, levofloxacin; SXT, trimethoprim-sulfamethoxazole; S, susceptible; R, resistant; U, undetermined; MbP, million base pairs; kbp, thousand base pairs.

$^b$ Time of collection was defined in days relative to the date of collecting the initial \textit{S. maltophilia} isolate in the case patient.

$^c$ Change in levofloxacin susceptibility investigated in this study.

$^d$ Inconsistent results were obtained in replicate.

and compared to whole-genome sequences of five control \textit{S. maltophilia} isolates (patients 2 to 6). All sequences were de novo assembled, i.e., without regard to reference assemblies. Table 1 summarizes the relative dates of collection, antimicrobial susceptibility results, and assembly statistics.

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served in an in vitro model strain created to study the MDR phenotype in 1997 (4). Using WGS and susceptibility testing, we can confirm that this SNV was the only variant to emerge and that it was sufficient to confer quinolone resistance in a clinical case. This underscores the need for clinicians to consider repeating DST during monotherapy if clinical signs suggest therapy failure.

smeT appears to play a central role in adaptive resistance to quinolones and other antibiotics effluxed by smeDEF, like tetracycline, chloramphenicol, erythromycin, and aminoglycosides. Since any mutation that inactivates this protein would be able to derepress smeDEF and confer resistance, smeT is under intense selective pressure in the presence of these drugs. In this study, we observed not only a deleterious SNV in the strain that displayed resistance (ISMMS2R) but also a premature stop codon in smeT in a strain that was already resistant at first isolation (ISMMS4). Certain nucleotide positions appear to be under greater selective pressure than others, as evidenced by our observation of the same mutation that occurred in D457R and a relative paucity of non-synonymous coding mutations in smeT observed among clinical smeT isolates (19). Since sustained overexpression of smeDEF is physiologically unfavorable (20), it is possible that pathogenic strains of S. maltophilia rely on natural diversity of mutations in the smeT locus to activate or deactivate smeDEF expression, allowing for rapid adaptation to antibiotic stress, though further study is needed.

Since resistance from a single SNV emerged during a short course of ciprofloxacin, clinicians should be cautioned about using quinolone monotherapy for S. maltophilia bacteremia, as highlighted in recent retrospective studies (21, 22). The wide variety of MDR phenotypes and unreliability of DST results have created uncertainty about appropriate treatment for S. maltophilia, but SXT remains the most common choice for monotherapy (1, 21, 22). SXT resistance in S. maltophilia is not known to be caused by efflux systems but has been linked to class 1 integrons and IS{sub}CR elements (1). This suggests that spontaneous resistance is less likely to emerge with SXT monotherapy, although a clinical trial comparing the two antibiotics is warranted (21, 22).

In conclusion, characterizing the full extent of genetic alterations that S. maltophilia utilizes to develop antibiotic resistance in vivo and improving genomic surveillance of clinical strains will help refine antibiotic selection criteria available to clinicians. Furthermore, this study highlights the utility of WGS for profiling the precise mutations underlying emerging antibiotic resistance in clinical cases of bacteremia.
Nucleotide sequence accession numbers. Complete genome sequences for ISMMS2, ISMMS2R, and ISMMS3 were deposited in DDBJ/EMBL/GenBank under accession numbers JZIU00000000, JZIV00000000, and JZTX00000000, respectively. Deposited sequences for ISMMS2 and ISMMS2R incorporate the Sanger corrected regions described above. Sequences for ISMMS4, ISMMS5, ISMMS6, and ISMMS7 were deposited as whole-genome shotgun projects at DDBJ/EMBL/GenBank under accession numbers JZIU01000000, JZIV01000000, and JZTX01000000, respectively, with the versions described in this paper at JZIU00000000, JZIV00000000, and JZTX00000000, respectively.

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REFERENCES