Extensively Drug-Resistant Pseudomonas aeruginosa Isolates Containing bla(VIM-2) and Elements of Salmonella Genomic Island 2: a New Genetic Resistance Determinant in Northeast Ohio

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Carbapenems are a mainstay of treatment for infections caused by *Pseudomonas aeruginosa*. Carbapenem resistance mediated by metallo-β-lactamases (MBLs) remains uncommon in the United States, despite the worldwide emergence of this group of enzymes. Between March 2012 and May 2013, we detected MBL-producing *P. aeruginosa* in a university-affiliated healthcare system in northeast Ohio. We examined the clinical characteristics and outcomes of patients, defined the resistance determinants and structure of the genetic element harboring the *bla*<sub>VIM</sub>-2 gene through genome sequencing, and typed MBL-producing *P. aeruginosa* isolates using pulsed-field gel electrophoresis (PFGE), repetitive sequence-based PCR (rep-PCR), and multilocus sequence typing (MLST). Seven patients were affected, who were hospitalized at three community hospitals, a long-term-care facility, and a tertiary care center; one of the patients died as a result of infection. Isolates belonged to sequence type 233 (ST233) and were extensively drug resistant (XDR), including resistance to all fluoroquinolones, aminoglycosides, and β-lactams; two isolates were nonsusceptible to colistin. The *bla*<sub>VIM</sub>-2 gene was identified as *bla*<sub>VIM</sub>-2 contained within a class 1 integron (In559), similar to the cassette array previously detected in isolates from Norway, Russia, Taiwan, and Chicago, IL. Genomic sequencing and assembly revealed that In559 was part of a novel 35-kb region that also included a Tn501-like transposon and *Salmonella* genomic island 2 (SGI2)-homologous sequences. This analysis of XDR strains producing VIM-2 from northeast Ohio revealed a novel recombination event between *Salmonella* and *P. aeruginosa*, heralding a new antibiotic resistance threat in this region’s healthcare system.

**Successful treatment of infections caused by *Pseudomonas aeruginosa*** depends on the early administration of effective antimicrobial therapy (1). Carbapenems have been among the most active antibiotics against *P. aeruginosa*; unfortunately, carbapenem-resistant *P. aeruginosa* is increasingly prevalent (2–4). Carbapenem resistance in *P. aeruginosa* is most often caused by mechanisms associated with antibiotic-mediated selection of mutants, such as loss of the OprD porin and overexpression of cephalosporinas and efflux pumps. Additionally, *P. aeruginosa* can acquire resistance through horizontal transfer of mobile genetic elements encoding enzymes that inactivate carbapenems, including metallo-β-lactamases (MBLs) (5).

Resistance mediated by MBLs, able to hydrolyze all β-lactams except monobactams, is an emerging problem among *P. aeruginosa* isolates in many countries of Asia, Europe, Africa, and South America (6–10). One of the MBLs of foremost global importance is the *Verona* integron-encoded metallo-β-lactamase (VIM). Currently, MBL-mediated carbapenem resistance is infrequent in the United States although national surveillance programs have detected *bla*<sub>VIM</sub>-2 in *P. aeruginosa* and other bacteria (11–17). At the local and regional levels, the early detection of MBL-producing bacteria by clinical microbiology laboratories and the molecular characterization of these bacteria can help inform efforts to prevent and control their dissemination.
MATERIALS AND METHODS

Selection of bacterial isolates. University Hospitals is an integrated health care system consisting of a tertiary care medical center, six community hospitals, and a long-term-care facility located in northeast Ohio, served by a central clinical microbiology laboratory that processes approximately 250,000 cultures a year. A laboratory-based surveillance program was established in 2007 to detect and characterize carbapenem resistance among Gram-negative bacteria across the hospital system (19). Clinical isolates of *P. aeruginosa* for which the imipenem or meropenem MIC was $\geq 4 \mu g/mL$ were screened for the presence of acquired carbapenemases using a modified Hodge test (mHT) with imipenem and for the presence of MBLs using imipenem-EDTA Etest strips (bioMérieux, Athens, GA). A 3-fold reduction in the imipenem MIC in the presence of EDTA triggered genetic testing for *bla* 

Molecular typing PFGE and rep-PCR. Molecular typing of *P. aeruginosa* isolates containing VIM-2 was performed with pulsed-field gel electrophoresis (PFGE) using the restriction enzyme SpeI for digestion and previously described analysis criteria (23, 24). Genetic similarity of VIM-2-harboring *P. aeruginosa* isolates was also investigated by repetitive-sequence-based-PCR (rep-PCR). Bacterial DNA was extracted using an MOBio UltraClean microbial isolation kit (MOBio, Carlsbad, CA) and amplified using a DiversiLab Pseudomonas primers. Amplicons were separated by electrophoresis on microfluidic chips and analyzed on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Resulting band patterns were compared via the Pearson correlation method, using DiversiLab software (bioMérieux). Isolates with $\geq 95\%$ similarity were considered genetically related (17).

MLST. Multilocus sequence typing (MLST) was performed on *P. aeruginosa* isolates harboring VIM-2 according to the method of Curran et al. (25). Seven housekeeping genes (*aaxA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*) were amplified by PCR and sequenced. Sequences were compared to existing alleles, and allelic numbers and sequence types (STs) were assigned according to the Pseudomonas aeruginosa public MLST database (http://pubmlst.org/paeruginosa/).

PCR amplification and sequencing of genes encoding β-lactamases and aminoglycoside resistance enzymes. Among isolates with a positive mHT, PCR amplification of *bla* 

Immunoblotting. In order to assess VIM-2 expression, rabbit polyclonal antibodies were raised against purified VIM-2-β-lactamase by New England Peptide and isolated from serum using protein G column purification (Sigma Genosys, The Woodlands, TX). Sample preparation, immunoblotting, and development were performed as previously reported (29, 30).

Mating experiments. To investigate the possibility of conjugal transfer of the *bla* 

Genome sequencing and assembly. Further genomic analysis of the *P. aeruginosa* wound isolate from case 1 was undertaken. Genomic DNA was extracted using a Wizard genomic DNA purification kit (Promega, Madison, WI). Genomic DNA was fragmented, and genomic libraries were prepared using an Ion Xpress Plus Fragment Library Kit (Life Technologies, Carlsbad, CA). Whole-genome sequencing was performed using the Ion Torrent Personal Genome Machine (PGM) platform per the manufacturer’s instructions (Life Technologies, Carlsbad, CA). Sequencing reads were assembled into a consensus de novo assembly using CLC genomic workbench software (version 5.5.1; CLC Bio, Aarhus, Denmark). Based on overlapping terminal regions, the contigs were assembled into supercontigs. The *bla* 

RESULTS

Clinical characteristics and antimicrobial susceptibility testing (AST). In the 2-year period of 2012 and 2013, the laboratory received 3,922 single-patient isolates of *P. aeruginosa*. Among these strains, the prevalence of carbapenem resistance was 20%. As part of our laboratory-based surveillance program, phenotypic testing with the mHT and imipenem/EDTA identified seven patients with MBL-producing *P. aeruginosa*, representing 0.9% of carbapenem-resistant *P. aeruginosa* isolates.

The initial isolate of MBL-producing *P. aeruginosa* (case 1) was detected in March 2012 from a wound in the foot of a 69-year-old man with type 2 diabetes mellitus who resided at a long-term-care facility; the patient also had asymptomatic bacteriuria with XDR *P. aeruginosa*. The mHT indicated carbapenemase production, and the imipenem MIC was lowered from $>256 \mu g/mL$ to 6 $\mu g/mL$ in the presence of EDTA. MBL-producing *P. aeruginosa* isolates were similarly detected in isolates from six additional patients (cases 2 to 7) between April 2012 and May 2013 (see Table S1 in the supplemental material). Case 4 had a bloodstream infection and died despite appropriate therapy with aztreonam and colistin. Case 5 was transferred to the tertiary care center from Qatar, in the Persian Gulf. Of note, case 1 was admitted to the same ward in a long-term-care facility where case 3 had resided until 4 days before. Cases 2, 4, 6, and 7 were all treated at the same community hospital, but only cases 6 and 7 had overlapping admissions. Cases 2, 6, and 7 had been residents or were discharged to a second long-term-care facility, which is associated with community hospital 2.

AST (Table 1) indicated that the nine isolates from these seven patients exhibited imipenem MICs of $>8 \mu g/mL$ and were resistant to all other β-lactams, as well as fluoroquinolones and aminoglycosides. Isolates from cases 1, 2 (second isolate), and 3 were additionally resistant to aztreonam. Isolates from cases 1 and 3 demonstrated intermediate resistance to colistin (MIC of 4 $\mu g/mL$), whereas all isolates were susceptible to polymyxin B.

Molecular typing, genetic analysis, and immunoblotting. Typing with rep-PCR revealed that all isolates shared $>95\%$ similarity (Fig. 1). PFGE performed on six of the nine isolates demonstrated that all isolates were closely related ($<3$ band differences) (data not shown). MLST classified all isolates as *P. aeruginosa* sequence type 233 (ST233). PCR detected *bla* 

Attempts to transfer the *bla* 

The nucleotide sequence of the *bla* 

"Antimicrobial Agents and Chemotherapy"
intI1 forward, 5’-GCAAGCGCCGCGACGGCCGAG-3’; tniC reverse, 5’-GAAACCAAGGTGGGCGATC-3’. Analysis of this molecular genetic element revealed two aminoglycoside-modifying enzyme (AME) genes: aacA7, a gene conferring resistance to amikacin and tobramycin, and aacC-A5, conveying resistance to gentamicin. Other AME or 16S rRNA methyltransferase genes were not detected. Steady-state protein expression assessed by Western blot analysis revealed that the VIM-2 β-lactamase was expressed at similar levels in all isolates tested (data not shown).

Structure of a blaVIM-2-harboring genomic island. Further analysis of the genetic environment of the VIM-2-producing P. aeruginosa wound isolate from case 1 revealed a genomic island harboring blaVIM-2 that is 34,954 bp in length and harbors 37 predicted ORFs (Fig. 2). This genomic island is integrated into the chromosome within a gene encoding the phosphate ABC transporter substrate-binding protein (PAS101 in P. aeruginosa PAO1). The whole region is bounded by 38-bp Tn3-like transposon-associated invert repeats (IRs), flanked by 6-bp duplications (TTTTCA) of the target sequence (target site duplications [TSD]). This recombinant element carries resistance to β-lactams (blaVIM-2 and blaOX4), aminoglycosides (aadA2, aacA7, and aacC-A5), tetracycline (tetA), chloramphenicol (cmlA6 and cmlA9), trimethoprim (dfrB5), antiseptics (qacE), sulfonamides (sul1), and mercury (mer operon) (Fig. 2).

The upstream 19-kb region is a Tn501-like transposon (~93% identical to Tn501) that includes genes encoding a transposase (tnpA) and a resolvase (tnpR), an inserted blaVIM-2-harboring class 1 integron, and a mer operon (Fig. 2). The tnpA (2,967 bp) and tnpR (561 bp) genes are 97.4% and 99.8% identical, respectively, to those of Tn501 from P. aeruginosa plasmid pVS1 (GenBank accession number Z00027). The Tn402-like class 1 integron, carrying an aacA7-blaVIM-2-dfrB5-aacC-A5 gene cassette array, is inserted downstream of the tnpR gene, generating a 5-bp duplication sequence (GCCCT) adjacent to the IR at the integrase end.

### TABLE 1 Antimicrobial susceptibility of VIM-2 producing Pseudomonas aeruginosa

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Date of isolation (mo-day-yr)</th>
<th>Specimen source</th>
<th>MIC (µg/ml)</th>
<th>Azeotremo</th>
<th>Meropenem</th>
<th>Colistin</th>
<th>Polymyxin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3-24-12</td>
<td>Foot wound</td>
<td>&gt;16</td>
<td>&gt;8</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5-4-12</td>
<td>Sputum</td>
<td>4</td>
<td>&gt;8</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5-16-12</td>
<td>Urine</td>
<td>&gt;16</td>
<td>&gt;8</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>9-1-12</td>
<td>Blood</td>
<td>4</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>9-17-12</td>
<td>Sputum</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3-12-13</td>
<td>Urine</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>5-7-13</td>
<td>Sputum</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Isolates were also resistant to piperacillin-tazobactam (MIC > 64/4 µg/ml), cefepime (MIC > 16 µg/ml), ceftazidime (MIC > 16 µg/ml), imipenem (MIC > 8 µg/ml), levofloxacin (MIC > 8 µg/ml), ciprofloxacin (MIC > 2 µg/ml), gentamicin (MIC > 8 µg/ml), tobramycin (MIC > 8 µg/ml), and amikacin (MIC > 32 µg/ml).

The sputum isolate from case 2 and isolates from cases 4, 5, 6, and 7 were interpreted as susceptible.

The wound isolate from case 1, both isolates from case 2, and isolates from cases 4, 5, 6, and 7 were interpreted as susceptible.

All isolates were interpreted as susceptible.

**FIG 1** Typing of VIM-2-producing Pseudomonas aeruginosa with rep-PCR demonstrates that cases 1 to 7 share ≥95% similarity. All isolates belong to sequence type 233. LTCF, long-term-care facility; Commun. Hosp., community hospital; Ctr, center.
INTRODUCTION

Recent studies have identified multiple cases of a multidrug-resistant (MDR) P. aeruginosa strain that has rapidly spread in the United States and Europe. This strain, designated as SGI2, is characterized by the presence of a large genomic island (GI) carrying several antimicrobial resistance (AMR) genes. The GI contains a class 1 integron that harbors genes for aminoglycoside (aacC-A5), trimethoprim (dfrB5), and meropenem (merE), among others. The integron also contains several transposons and integrons, which contribute to the horizontal gene transfer of AMR genes.

METHODS

To investigate the genetic relatedness and spread of the SGI2 strain, we analyzed a collection of P. aeruginosa isolates from various geographical locations. We performed genotypic and phenotypic characterization, including antimicrobial susceptibility testing and molecular typing methods such as pulsed-field gel electrophoresis (PFGE) and pulsed-field gel electrophoresis (PGE). We also used whole-genome sequencing (WGS) to compare the genetic content of the SGI2 strain with those from other regions.

RESULTS

We found that the SGI2 strain shares a high degree of genetic similarity with isolates from the United States, Europe, and Asia. The GI carrying the integron is highly conserved among these isolates, suggesting a common origin. Additionally, we identified multiple genetic markers associated with the spread of this strain, including specific integron and transposon arrays.

DISCUSSION

The emergence of SGI2ean 10 multidrug-resistant (MDR) P. aeruginosa strain that has rapidly spread in the United States and Europe. This strain, designated as SGI2, is characterized by the presence of a large genomic island (GI) carrying several antimicrobial resistance (AMR) genes. The GI contains a class 1 integron that harbors genes for aminoglycoside (aacC-A5), trimethoprim (dfrB5), and meropenem (merE), among others. The integron also contains several transposons and integrons, which contribute to the horizontal gene transfer of AMR genes.

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DISCUSSION

The emergence of SGI2
Further in-depth analyses of our strains revealed that the bla-VIM-2 gene was located in a complex 35-kb genomic island, consisting of integron In559 embedded in a Tn501-like transposon, and located in the chromosome of _P. aeruginosa_. Adjacent to this structure we found genetic elements homologous to SG12, a large genetic element first seen in _Salmonella_. Horizontal gene transfers between _Salmonella_ and other nonfermenters have been previously documented in clinical isolates, but none of these transfers resulted in a genetic element with the size and complexity of the one we found (42). Mobile genetic elements similar to those of _Salmonella_ found in our _P. aeruginosa_ strain have also been identified in the chromosome of _Acinetobacter baumannii_ (43). Our finding raises concerns about recombination events between bacterial species of diverse clinical, animal, and environmental origins that may result in the propagation of antibiotic resistance determinants (44). _Salmonella enterica_ carrying the VIM-2 MBL has been detected in Morocco, where the multidrug-resistant (MDR) _S. enterica_ serovar Kentucky is prevalent (45).

The detection of an international clone of VIM-2-producing _P. aeruginosa_ in different components of a health care system in our region heralds the potential threat of widespread dissemination of MBLs in northeast Ohio and beyond. The presence of a single clone and the epidemiological association with patients affected by multiple comorbidities and heavily exposed to health care are reminiscent of outbreaks of two other frequently carbapenem-resistant species, _A. baumannii_ and _Klebsiella pneumoniae_, which emerged in the United States during the past decade (46, 47). The dissemination of these highly resistant pathogens underscores the vulnerability of our patients and health care system, made more urgent by the lack of effective antibiotic therapies and the current crisis in antibiotic development (19, 48).

At present, disciplined adherence to infection control practices and prudent use of antibiotics remain the best strategies to prevent the emergence of VIM-producing _P. aeruginosa_. Although efforts and resources in this regard are increasingly deployed in the acute-care setting, significant challenges are faced in community and long-term-care facilities (49). This report highlights the important role of laboratory surveillance enhanced with detailed genetic characterization in identifying emerging resistant phenotypes at the local and regional levels (50). These efforts led to the identification of a novel recombination event resulting from a Tn501-like transposon and SG12, expanding our understanding of the molecular epidemiology of MBL-producing _P. aeruginosa_.

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