

# Extensively Drug-Resistant *Pseudomonas aeruginosa* Isolates Containing *bla*<sub>VIM-2</sub> 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- $\beta$ -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 health care 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>MBL</sub> 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 that 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  $\beta$ -lactams; two isolates were nonsusceptible to colistin. The *bla*<sub>MBL</sub> gene was identified as *bla*<sub>VIM-2</sub> 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 health care 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 cephalosporinases and efflux pumps. Additionally, *P. aeruginosa* can acquire resistance through horizontal transfer of mobile genetic elements encoding enzymes that inactivate carbapenems, including metallo- $\beta$ -lactamases (MBLs) (5).

Resistance mediated by MBLs, able to hydrolyze all  $\beta$ -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- $\beta$ -lactamase (VIM). Currently, MBL-mediated carbapenem resistance is infrequent in the United States although national surveillance programs have detected *bla*<sub>MBLs</sub> 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.

Here, we report the dissemination of *P. aeruginosa* isolates containing VIM-2 in patients from community hospitals in northeast Ohio. These isolates displayed an extensively drug resistant (XDR) phenotype, leaving few, if any, treatment options (18). As this was a “sentinel event” in our region, our objective was to define the molecular characteristics of the outbreak strain and to place it in the global context of MBL-producing *P. aeruginosa*. These analyses led us to discover a genetic element that represents a novel recombination event between elements found in *P. aeruginosa* and *Salmonella*.

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## 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  $\geq 4$   $\mu\text{g/ml}$  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*<sub>MBLs</sub> (20, 21). Antimicrobial susceptibility testing (AST) was performed using the MicroScan System (Siemens, Tarrytown, NY), and results were interpreted according to Clinical and Laboratory Standards Institute guidelines (22).

**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 *Spe*I 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* kit. 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 (*acsA*, *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  $\beta$ -lactamases and aminoglycoside resistance enzymes.** Among isolates with a positive mHT, PCR amplification of *bla*<sub>IMP</sub>, *bla*<sub>KPC</sub>, *bla*<sub>NDM</sub>, and *bla*<sub>VIM</sub> genes was performed (26). We also tested for the presence of genes encoding aminoglycoside-modifying enzymes (AME) and 16S rRNA methyltransferases using validated primers and PCR conditions as described previously (27, 28). Amplicons were sequenced at Molecular Cloning Laboratories (MCLAB; San Francisco, CA).

**Immunoblotting.** In order to assess VIM-2 expression, rabbit polyclonal antibodies were raised against purified VIM-2  $\beta$ -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 conjugative transfer of the *bla*<sub>VIM-2</sub> gene, *bla*<sub>VIM-2</sub>-containing *P. aeruginosa* isolates susceptible to rifampin were selected for by overnight growth in LB medium along with rifampin-resistant *Escherichia coli* J-53 or *P. aeruginosa* PAO1 (American Type Culture Collection, Manassas, VA). After overnight culture, cells were plated on Mueller-Hinton agar plates containing 100 mg/liter ampicillin and 100 mg/liter rifampin. Colonies that subsequently grew were replated on the same selective medium and screened by PCR for the presence of *bla*<sub>VIM</sub>.

**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 Technolo-

gies, 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*<sub>VIM</sub>-harboring supercontig was extracted, and the open reading frames (ORFs) were predicted and annotated using the RAST ([rast.nmpdr.org](http://rast.nmpdr.org)) server (31). The IS Finder database ([www-is.biotoul.fr](http://www-is.biotoul.fr)) was used to identify the names/numbers of insertion elements that were found using the RAST server. The structure of the *bla*<sub>VIM</sub>-harboring genomic island was confirmed by long-range PCRs using internal and overlapping primers.

**Nucleotide sequence accession number.** The nucleotide sequence of the *bla*<sub>VIM</sub>-harboring genomic island was deposited in GenBank under accession number [KJ463833](https://www.ncbi.nlm.nih.gov/nuclot/KJ463833).

## 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  $>256$   $\mu\text{g/ml}$  to 6  $\mu\text{g/ml}$  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  $>8$   $\mu\text{g/ml}$  and were resistant to all other  $\beta$ -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  $\mu\text{g/ml}$ ), 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*<sub>VIM-2</sub> in all MBL-producing *P. aeruginosa* isolates, while other carbapenemase genes were absent.

Attempts to transfer the *bla*<sub>VIM-2</sub> gene to *P. aeruginosa* PAO1 or *E. coli* J-53 were unsuccessful. A 3.2-kb Tn402-like class 1 integron fragment was amplified in all isolates with the following primers:

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

Case no.	Date of isolation (mo-day-yr)	Specimen source	MIC (μg/ml) <sup>a</sup>			
			Aztreonam <sup>b</sup>	Meropenem	Colistin <sup>c</sup>	Polymyxin B <sup>d</sup>
1	3-24-12	Foot wound	>16	>8	2	2
	3-24-12	Urine	>16	>8	4	2
2	5-4-12	Sputum	4	>8	2	2
	5-28-12	Sputum	>16	>8	2	1
3	5-16-12	Urine	>16	>8	4	2
4	9-1-12	Blood	4	8	2	2
5	9-17-12	Sputum	8	8	2	2
6	3-12-13	Urine	8	8	2	2
7	5-7-13	Sputum	8	8	2	2

<sup>a</sup> 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).

<sup>b</sup> The sputum isolate from case 2 and isolates from cases 4, 5, 6, and 7 were interpreted as susceptible.

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

<sup>d</sup> All isolates were interpreted as susceptible.

*intI1* forward, 5=GCAAGCGCCGCGACGCGCCGAG-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 *bla*<sub>VIM-2</sub>-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 *bla*<sub>VIM-2</sub> 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 (PA5101 in *P. aeruginosa* PAO1). The whole region is bounded by 38-bp Tn3-like trans-

poson-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 (*bla*<sub>VIM-2</sub> and *bla*<sub>OXA-4</sub>), aminoglycosides (*aadA2*, *aacA7*, and *aacC-A5*), tetracycline [*tetA*(G)], chloramphenicol (*cmlA6* and *cmlA9*), trimethoprim (*dfrB5*), antiseptics (*qacEΔ1*), sulfonamides (*sul1*), and mercury (a *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 *bla*<sub>VIM-2</sub>-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*-*bla*<sub>VIM-2</sub>-*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

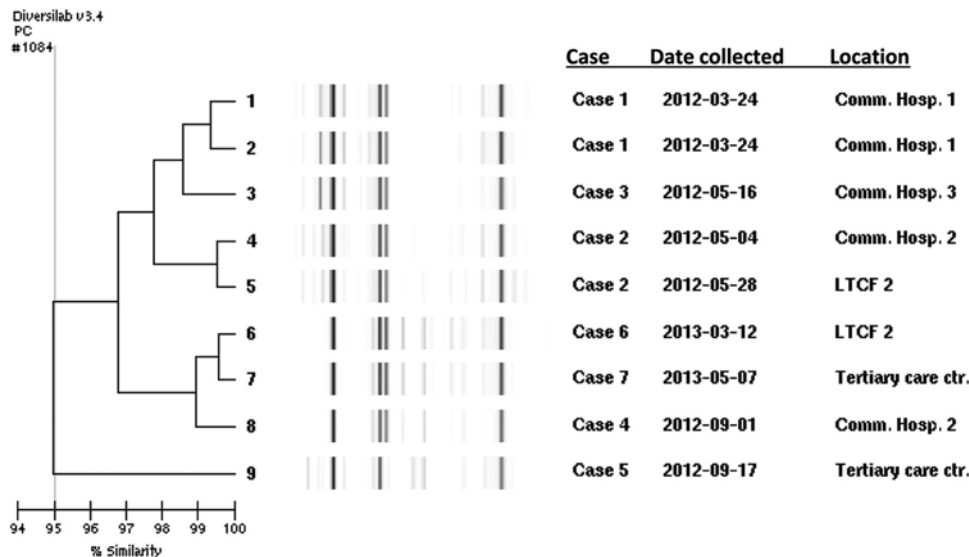
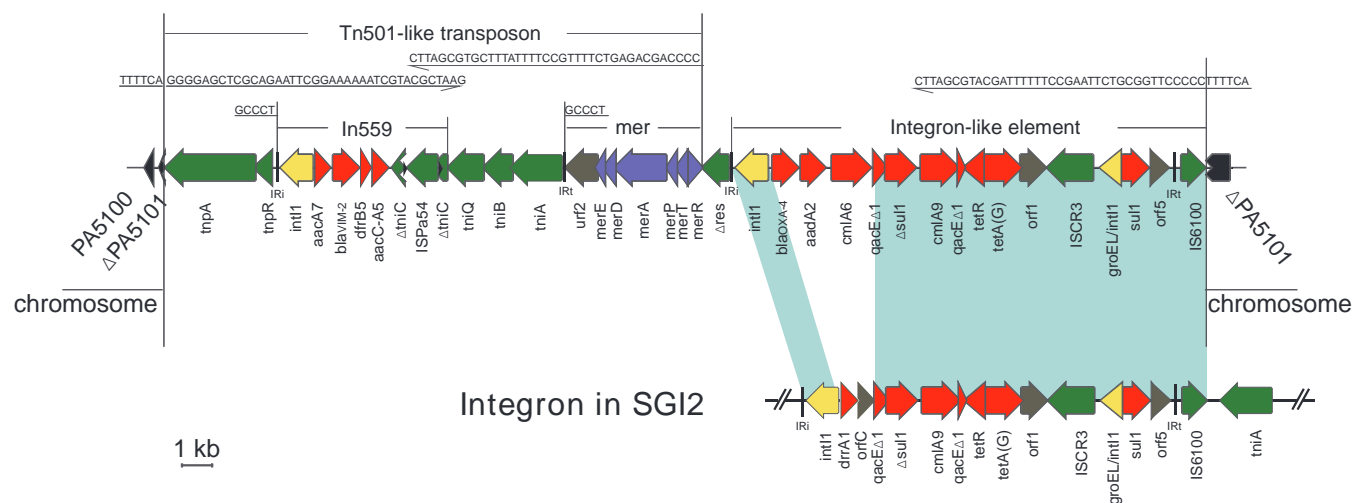


FIG 1 Typing of VIM-2-producing *Pseudomonas aeruginosa* with rep-PCR demonstrates that cases 1 to 7 share ≥95% similarity. All isolates belong to sequence type 233. LTCF, long-term-care facility; Commun. Hosp., community hospital; Ctr., center.





**FIG 2** Tn501-like transposon containing *bla<sub>VIM</sub>* proximal to an integron-like element very similar to SGI2 (GenBank accession no. [KJ463833](#)) from *Salmonella enterica* subsp. *enterica* serovar Emek (GenBank accession no. [AY963803](#)). Open reading frames (ORFs) are represented by arrows. The genes located in the *Pseudomonas aeruginosa* chromosome are illustrated with black arrows. The genes encoding transposase or resolvase, or within IS elements are indicated by green arrows, and the genes in the *mer* operon are represented with purple arrows. Shading with light blue indicates >99.5% nucleotide identity. Antimicrobial resistance genes are shown with red arrows, and the *int1* integrase gene is shown with yellow arrows. The target duplicated sequences (TTTCA) and In559 (GCCCT) are in bold and underlined. The three 38-bp invert repeats (IRs) are shown in italics. Single-arrow lines beneath the IRs indicate the direction of the repeats. The IR at the integrase end (IRi) and the IR at the transposon end (IRt) are indicated with vertical black bars. Small black arrowheads that flank ISPa54 in *tniC* denote its 5-bp (TTAGG) direct repeats.

(IRi) and the IR at the transposon end (IRt). BLAST results show only four nucleotide differences (>99.9% identity) with the partial sequence of integron In559 (GenBank accession number [KC821786](#)) and a novel IS element, designated ISPa54, present in the *tniC* gene. The *mer* operon (4.5 kb), located downstream of the *bla<sub>VIM</sub>*-harboring integron, is nearly identical (>99.9% identity) to that from a *P. aeruginosa* strain C79 genomic island (GenBank accession number [JF826498](#)), suggesting a hybrid transposon structure.

The downstream region is a 16-kb integron-like element, with its overall structure resembling that of the integron-like element identified in *Salmonella* genomic island 2 (SGI2) in *Salmonella enterica* subsp. *enterica* serovar Emek (32). The only difference between the two integrons is that in the isolate from this study, a *bla<sub>OXA-4</sub>*-*aadA2*-*cmlA6* gene array replaces the *dfrA1*-*orfC* gene array found in SGI2. The rest of this region (including the 5= conserved segment and the fragment from *qacEΔ1* to IS6100 [12.5 kb]) shows >99.9% identity to that of the SGI2. There is also an extra IRt close to IS6100 in an In4-like structure.

A 913-bp fragment, located between the above-described Tn501-like transposon and the 16-kb integron-like element, is identical to part of *P. aeruginosa* strain C plasmid pKLC102 transposon TNCP23 (GenBank accession number [AY257539](#)), carrying a putative resolvase gene, *res*. Interestingly, the *res* gene of the strain in this study was disrupted by the *mer* operon at 826 bp downstream of the start codon. A third 38-bp Tn3-like transposon IR was found immediately abutting this truncated *res* gene. PCR assays confirmed the presence of the same genomic island in all isolates.

## DISCUSSION

Through laboratory-based surveillance, we uncovered the presence of genetically related XDR *P. aeruginosa* harboring VIM-2 in seven patients from a regional hospital system in northeast Ohio. Alarmingly, the XDR phenotype expressed by some of these iso-

lates precluded any reliable antibiotic treatment since they even displayed intermediate resistance to colistin, an “agent of last resort.” Aztreonam, a monobactam usually stable against MBLs, was not consistently active either. Patients who were affected had multiple comorbidities, endured prolonged colonization, required long-term care, and, in one instance, had a lethal outcome from a bloodstream infection. The epidemiological link among these genetically related isolates appears to be admission to a community hospital and residence in long-term-care facilities in northeast Ohio, with the exception of a patient who was transferred to the tertiary medical center from Qatar. After the detection of MBL-producing *P. aeruginosa* by the clinical microbiology laboratory, enhanced infection control precautions and contact isolation were implemented. Despite ongoing surveillance 12 months after the last case, no additional cases have been detected.

All *P. aeruginosa* isolates in this report belonged to ST233 and contained *bla<sub>VIM-2</sub>* in the In559 cassette array. The gene array in this class 1 integron is almost identical to genes characterized in *P. aeruginosa* isolates from an outbreak in 2003 at John H. Stroger, Jr., Hospital of Cook County in Chicago, IL (isolate 7052; GenBank accession number [AY943084](#)) (12). The same genetic element was detected in Norway (GenBank accession number [FM165436](#)) in *P. aeruginosa* ST233 imported from Ghana (33), in a *P. aeruginosa* isolate from Taiwan (isolate S1-36) (34), and in *P. aeruginosa* ST235 from Russia (GenBank accession number [DQ522233](#)). We note that *P. aeruginosa* ST233 and ST235 do not share any alleles (<http://pubmlst.org/paeruginosa>). Recently, the widespread dissemination of *P. aeruginosa* ST235 harboring *bla<sub>VIM-2</sub>* in a similar array was described in Russia and its neighboring region, as well as in several Asian countries (35, 36). Other high-risk clones of VIM-producing *P. aeruginosa* have also spread successfully (37); for instance, *P. aeruginosa* ST175 harboring VIM-2 has been detected in Spain, France, and Central Europe (23, 38–41).

Further in-depth analyses of our strains revealed that the *bla*<sub>VIM-2</sub> 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 SGI2, 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 SGI2, expanding our understanding of the molecular epidemiology of MBL-producing *P. aeruginosa*.

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