Short Communication

Identification of a Novel Metallo- β -Lactamase VIM-28 Located within Unusual Arrangement of Class 1 Integron Structure in *Pseudomonas aeruginosa* Isolates from Egypt

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SUMMARY: Two multidrug-resistant *Pseudomonas aeruginosa* strains harboring *bla*_{VIM} were isolated from a hospital in Egypt and were indistinguishable by pulsed-field gel electrophoresis. Sequence analysis revealed a novel VIM β -lactamase, VIM-28. Additional sequencing of integron revealed that the *bla*_{VIM-28} gene cassette had an unusual arrangement of class 1 integron structure, located directly downstream of the integrase gene “*intI1*” and oriented divergently from it. The new organization of integron also comprised the *aacA7* and *smr-2* gene cassettes in that order. A complete *ISPa21* containing a *tnpA* gene flanked by two 13-bp inverted repeats was located directly upstream of the 3'-CS conserved region of the integron containing *qacE Δ 1/sul1* genes. The *bla*_{VIM-28}-containing class 1 integron was found to have a chromosomal origin in both strains. In conclusion, the presence of a new variant of *bla*_{VIM}, *bla*_{VIM-28}, on a new organization of class 1 integron having *ISPa21* increases the possibility of dissemination of resistance genes within the integron in *P. aeruginosa* among hospitalized patients in Egypt.

Pseudomonas aeruginosa is an important opportunistic pathogen of humans. It is notorious for its natural resistance to many antibiotics and quick ability to develop resistance to others (1,2). Carbapenem, a β -lactam highly resistant to most β -lactamases, is considered as the last resort for many bacterial infections. During the last 20 years, there has been an increasing concern over the spread of carbapenem resistance, particularly with no new antibiotics in the pipeline to combat strains resistant to carbapenems (3). Several mechanisms may contribute to carbapenem resistance in *P. aeruginosa*, such as impaired outer membrane porins, upregulation of efflux pumps, overexpression of extended-spectrum chromosomally encoded AmpC cephalosporinase, and production of enzymes that hydrolyze carbapenems (4). Metallo- β -lactamase (MBL), OXA-type carbapenemase, and class A carbapenemases, including KPC, SME, IMI, NMC-A, and GES, are examples of enzymes that have the ability to hydrolyze carbapenems (4,5). The genes encoding MBLs are mostly located as cassettes in integron or integron-like structures and are incorporated in the chromosome or on plasmids (6). Although several types of MBLs such as IMP, VIM, SPM, GIM, SIM, KHM, AIM, DIM, and NDM have been identified, IMP, NDM, and VIM are the most common types of MBLs worldwide (6,7). El-Kholy et al. (8) reported that 56.3% of *P. aeruginosa* isolates collected between 2009 and 2010 from three Cairo University hospitals in Egypt were resistant to imipenem. The present study investigated the mechanisms of carbapen-

em resistance in *P. aeruginosa* isolates from a hospital in Egypt.

Two multidrug-resistant *P. aeruginosa* strains (435 and 1698) were isolated between 2009 and 2010 from different patients in a hospital in Egypt. The susceptibility of both the *P. aeruginosa* strains to several antimicrobial agents, including imipenem, meropenem, cefepime, ceftazidime, aztreonam, piperacillin, piperacillin/tazobactam, cefotaxime, amikacin, gentamicin, tobramycin, ciprofloxacin, and levofloxacin, was assessed by the minimum inhibitory concentration (MIC) agar dilution method according to the Clinical and Laboratory Standards Institute guidelines (9) and by the Vitek 2 automated system. The susceptible strains, *Escherichia coli* ATCC25922 and *P. aeruginosa* 27853, were used as quality control strains to monitor the accuracy of MIC. Both the strains were resistant to all the antimicrobial agents tested and had the same resistance profile. Pulsed-field gel electrophoresis (PFGE) was conducted to determine the clonal relatedness of the *P. aeruginosa* isolates, as reported previously (10). *P. aeruginosa* strain PAO1 was used as the reference strain. The band patterns were considered to be related as per the recommendation of Tenover et al. (11). PFGE revealed that the two isolates were indistinguishable and were clonally unrelated to the susceptible strains of *P. aeruginosa* isolated from the same hospital.

Amplification of the *bla*_{VIM} gene was performed by real-time PCR using primer pair VIMF (5'-GGTGTTTGGTCGCATATCGC-3') and VIMR (5'-CCATTCAGCCAGATCGGCATC-3'). The reaction was performed in a 50- μ l volume comprising 0.25 μ l (7 pmol) of each primer, 2 μ l of DNA template, 2.5 μ l of EvaGreen dye, 5 μ l of Q-solution, and 25 μ l of 2 $\times$ Rotor-Gene PCR Master Mix, which includes HotStarTaq Plus DNA Polymerase, Rotor-Gene PCR Buffer, and dNTP mix (Qiagen, Hilden, Germany). PA23sRTF and R and

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ENTBC 50sL17F and R primers were used as internal controls and kept in a separate tube in the same real-time PCR run. The reaction conditions were as follows: 95°C for 15 min for the activation of the HotStarTaq *Plus* DNA Polymerase, followed by 35 cycles at 94°C for 30 s each (denaturation), 50°C for 90 s (annealing), and 72°C for 90 s (extension and fluorescence data collection). The PCR products were then subjected to a high-resolution melting cycle (70°C–99°C) with optimization of the dye acquired. The two resistant isolates (435 and 1698) were detected to harbor *bla*_{VIM} gene and were positive for MBL production in the EDTA-disk synergy test (12).

The genetic context of the *bla*_{VIM} gene was initially assessed with primers designed on the basis of the 5'- and 3'-conserved segments (CSs) of class 1 integrons (Table 1 and Figure 1). PCR reactions were performed in a 50-μl volume comprising 0.25 μl (1 unit) of PlatinumR *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA), 1× HiFi PCR buffer, 200 μM MgSO₄, 2 mM dNTP, 25 pmol of each primer, and 2 μl of DNA template extracted as mentioned above. The amplification reactions were performed under the following cycling conditions: 94°C for 5 min; 25 cycles at 94°C for 60 s, 50°C for 30 s, and 68°C for 120 s; and 68°C for 7 min, except for the PCR reaction utilizing the VIMF2 and *sul*IR primer pair, for which following cycling conditions were employed: 94°C for 5 min; 30 cycles at 94°C for 60 s, 48°C for 30 s, and 68°C for 180 s; and 68°C for 7 min. The sequence analysis comparisons were initially established by searching the NCBI database with the BLAST program (<http://www.ncbi.nlm.nih.gov>) and were accurately determined by DNA Baser V2.9 software for overlapped multi-sequence alignments and contig editing. Sequence analysis

of *bla*_{VIM} using the primer pair flanking the gene and *intI1* (VIM-*intI1*F and VIM-*intI1*R) revealed a novel variant of the gene. The deduced amino acid sequence of the enzyme showed one amino acid change in VIM-26 (S205R) and VIM-4 (H201L), and both amino acid changes in VIM-1. This change in the deduced amino acid sequence of the enzyme was not within its active site (15). The novel variant of this enzyme was designated as VIM-28 following the nomenclature by Jacoby and Bush (<http://www.lahey.org/Studies/>).

To characterize the *bla*_{VIM}-containing class 1 integron, PCR mapping and sequencing of both the strains revealed that the *bla*_{VIM-28} cassette is located directly downstream of the integrase gene *intI1* and oriented divergently from it. The class 1 integron also carries two other resistance gene cassettes, *aacA7* (coding for a 6'-Naminoglycoside acetyltransferase II) and *smr-2* (coding for a small multidrug resistance protein) (Figure 1). The class 1 integron is uncommonly disrupted by the insertion sequence *ISPa21*, which is composed of a putative transposase gene (*tnpA*) flanked by two 13-bp inverted repeats, IRR (5'-ATGGACTCTCCCC-3') and IRL (5'-GGGGAGAGTCCAT-3'). The insertion sequence is transcribed divergently from the three resistance genes within the class 1 integron (Figure 1). The nucleotide sequence data reported in this study have been submitted to the GenBank database under the accession no. JF900599.

The plasmid extraction protocol for the extraction of large plasmids (16) was used to investigate the presence of plasmids in *P. aeruginosa* VIM-containing isolates. No plasmid DNA was detectable by agarose gel electrophoresis in four plasmid preparation trials from both strains. The transfer of imipenem resistance from *P. aeruginosa* VIM-containing isolates as donors was at-

Table 1. Primers for PCR mapping of *bla*_{VIM}-containing integron-like structures

Primer	Nucleotide sequence (5' to 3')	Target or place in the integron	Reference
VIM- <i>intI1</i> F	GGTAGTGAATCCGCCAGG	Sequence of area between <i>intI1</i> and <i>bla</i> _{VIM-28}	This study
VIM- <i>intI1</i> R	GCAAAAGTCCCGCTCCAACG	Sequence of area between <i>intI1</i> and <i>bla</i> _{VIM-28}	This study
<i>attI1</i> F	GGCATCCAAGCAGCAAG	Within <i>attI1</i> site	13
<i>intI1</i> F	GACGATGCGTGGAGACC	For 5'-CS end of class 1 integron and within <i>intI1</i> gene	14
R1I	AAGCAGACTTGACCTGA	In the 3'-CS end conserved segment of class 1 integron	13
<i>qacEΔ1</i> R	CGGATGTTGCGATTACTTCG	Within <i>qacEΔ1</i> gene	This study
<i>sul</i> IR	GGCATGATCTAACCTCGGTC	Within <i>sul</i> gene	This study
<i>smr-2</i> F	CGTCGGCGTTCTTTACTACG	Within <i>smr-2</i>	This study
VIMF2	GCACACAAAATCGCTCAGTCG	At the 3'-end of <i>bla</i> _{VIM-28}	This study

Fig. 1. Schematic representation of the class 1 integron structure with *bla*_{VIM-28} in *P. aeruginosa* strains 435 and 1698. The gene cassettes are represented by boxed arrows indicating the transcriptional orientation. IRR and IRL motifs of *ISPa21* are indicated by asterisks. Arrows under the figure represent the location of primers used for PCR mapping of class 1 integron.

tempted by filter mating (17) with *E. coli* J53 as the recipient strain. The initial donor/recipient ratio was 1:5 or 1:10. The transconjugants were selected on MacConkey agar containing ceftazidime (4 µg/ml). Despite several conjugation trials, the conjugation experiments failed to demonstrate the occurrence of conjugative transfer of *bla*_{VIM-28} from *P. aeruginosa* 435 or 1698 to *E. coli* J53. Southern blotting was performed following PFGE of I-CeuI (the rare-cutting restriction enzyme)- and *Spe*I-digested genomic DNA of *P. aeruginosa* 435 and 1698 isolates. The DIG DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany) was used to label the *bla*_{VIM} probe and for hybridization according to the manufacturer's recommendations. The *bla*_{VIM} probe was prepared using the VIMF and VIMR primer pair. Southern blot results revealed that *bla*_{VIM-28} carried on the class 1 integron is apparently incorporated within the chromosome (data not shown).

According to my knowledge, the only study that explained the acquisition of VIM in *P. aeruginosa* isolates from Egypt was a case of a Hungarian tourist initially hospitalized in Egypt and later in Hungary (18); however, no further characterization of the genetic element harboring *bla*_{VIM-2} was performed in that study. Although integrons themselves are not mobile, several transposon-related structures have been found in class 1 integrons, such as IS*Pa21* containing a *tnpA* in the present study, Tn402 from Argentina (19), and Tn5090 from Portugal (20). In both previous studies (19,20), *bla*_{VIM-2} was isolated from *Pseudomonas putida*, an important reservoir for antibiotic resistance genes specific to *P. aeruginosa*, despite its uncommon association with human infections within the hospital environment.

The current finding of the unusual class 1 integron containing *bla*_{VIM-28}, *aacA7*, and *smr-2* gene cassettes accompanied by a complete IS*Pa21*-containing *tnpA* gene, flanked by two 13-bp inverted repeats, IRR and IRL, is the first report of such an arrangement in *P. aeruginosa*. This insertion sequence, when associated with different antibiotic resistance genes, may play an important role in their dissemination among diverse genera of bacteria (20). Recently, IS*CR1* was reported in a *bla*_{VIM-4}-containing class 1 integron downstream of the *qacEΔ1/sulI* genes in the 3'-CS conserved region of the integron from *Enterobacter cloacae* isolated from Egypt (21).

In conclusion, the present study confirms the emergence of VIM-producing *P. aeruginosa* in Egypt and raises the concern of its spread to other *P. aeruginosa* infecting hospitalized patients. VIM-28 represents a further addition to the list of VIM enzyme variants, being described from *P. aeruginosa* isolates from a Middle East country. The novel organization of class 1 integron described here adds to our understanding of multiple possible ways in which MBLs can spread in various bacterial genera.

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Conflict of interest None to declare.

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