Evolution of an integron carrying $\text{bla}_{\text{VIM-2}}$ in Eastern Europe: report from the SENTRY Antimicrobial Surveillance Program

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As part of the SENTRY Antimicrobial Surveillance Program, an imipenem-resistant Pseudomonas aeruginosa strain (81-11963A) was isolated from the blood culture of a female neonate institutionalized at the local children’s hospital in Warsaw, Poland. Cloning of an imipenem resistance determinant revealed it to be a VIM-2 metallo-β-lactamase, but sequence analysis of DNA adjacent to $\text{bla}_{\text{VIM-2}}$ revealed it to have a unique gene context. Downstream of the $\text{bla}_{\text{VIM-2}}$ gene resides an aacA4 gene encoding the AAC(6′)- Ib amino-glycoside acetyltransferase. The integron containing $\text{bla}_{\text{VIM-2}}$ shows high similarity to that reported from In58 in France but was novel in that it possessed a gene cassette with a 59 truncated base element only 19 base pairs (bp) long, consisting of a conserved core site and an inverse core site separated by only 5 bp. This appears to be the first report of a metallo-β-lactamase gene arising from a pathogenic strain in Eastern Europe.

Keywords: metallo-β-lactamases, Poland, Pseudomonas aeruginosa

Introduction

In recent years reports of clinical isolates of Pseudomonas aeruginosa that are resistant to virtually all β-lactams have become more common. These isolates have been shown to produce metallo-β-lactamases, enzymes that are usually zinc dependent. These metal ions co-ordinate water molecules that serve as nucleophiles, which attack and break the cyclic amide bond of the β-lactam ring, rendering the antibiotic biologically inactive. The enzyme types are IMP, VIM and the recently described SPM β-lactamase.¹ VIM-type enzymes have been found in P. aeruginosa isolated in Europe and Southeast Asia, and VIM-1 in Italy,²,³ VIM-2 in France,⁴,⁵ Greece,⁶ Italy,⁷ Korea⁸ and Spain.⁹ VIM-2 and VIM-3 have been found in isolates from Taiwan and VIM-4 in isolates from Greece.¹⁰ Most genes encoding IMP- and VIM-type metallo-β-lactamases are found on gene cassettes of class 1 integrons. Integrons are genetic elements that possess a specific recombination site, attI1, into which resistance genes, in the form of gene cassettes, can be inserted by site-specific recombination.

As part of the SENTRY Antimicrobial Surveillance Program, an imipenem-resistant isolate of P. aeruginosa (strain 81-11963A) was recovered from a blood culture of a female neonate from the local children’s hospital in Warsaw, Poland. Here we report the genetic characterization of the carbapenem resistance determinant from P. aeruginosa 81-11963A. We also offer an explanation as to how this new integron may have arisen from In58. The isolation of P. aeruginosa 81-11963A represents the first reported appearance of the metallo-β-lactamase VIM-2 in Eastern Europe.

Materials and methods

Bacterial strains and plasmids

P. aeruginosa 81-11963A was a clinical isolate from Warsaw, Poland. Escherichia coli strain DH5α [supE44 ΔlacU169 (φ80lacZAM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1] was used as the host strain to express the cloned β-lactamase gene. Positive controls for the IMP- and VIM-type metallo-β-lactamases were P. aeruginosa strains carrying the respective genes. The genomic library was generated in the cloning vector pK18 as previously described.¹

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**Novel bla\textsubscript{VIM} integron in Pseudomonas aeruginosa**

**Materials**

Antimicrobial agents used were ceftazidime (GlaxoSmithKline, Worthing, UK) and kanamycin (Sigma Chemical Co., St Louis, MO, USA). PCR primers were purchased from Sigma-Genosys Ltd (Pampsisford, UK). General reagents for DNA manipulation were obtained from Invitrogen (Groningen, The Netherlands). All other reagents were obtained from Sigma Chemicals Co. or BDH (both of Poole, UK).

**Determination of MICs**

Mid-log phase grown cultures (optical density of 0.6 at 600 nm) were diluted to 1/10\(^4\) in water. Ten microlitres from each dilution was spotted onto Mueller--Hinton agar (BBL; Becton Dickinson, Oxford, UK), containing serial dilutions of the appropriate agent, using a multipoint inoculator. After 24 h incubation at 37°C, the MIC was noted as the lowest concentration of antimicrobial that inhibited the growth in those dilutions which, when inoculated onto nutrient agar containing no drug, gave rise to single colonies.

**PCR screening for bla\textsubscript{VIM} and bla\textsubscript{IMP} metallo-\(\beta\)-lactamase genes**

For amplification using primers based on the conserved regions of the imp and vim genes, PCR was performed using AB-gene Expand Hi-Fidelity master mix containing a mix of \(Pfu\) non-proof reading Taq polymerases and dNTPs. Primers used to detect \(vim\textsubscript{imp}\) genes were:

- VIM forward, TTATGGAGCAGCAAGCAGTG
- VIM reverse, CCTTATTC; and
- IMP reverse, GCTGCAACGACTTGTTAG.

Primers were used at 10 pM concentrations and 1 µl of bacterial culture at density OD\(1\) at 600 nm was used as template. Cycling parameters were:

- 95°C for 1 min and extension 68°C for 1 min, annealing at 45°C for 5 min and ending with a 5 min incubation at 68°C.

**Recombinant DNA methodology and DNA sequencing analysis**

Genomic DNA was isolated from \(P.\ aeruginosa\) strain 81-11963A by the cetyltrimethylammonium bromide method and gene libraries were created using size fractionated DNA as described previously. The ligation mixture was subsequently dialysed and used to transform \(E. coli\) DH5\(\alpha\) to ceftazidime resistance by electroporation. The clone containing the metallo-\(\beta\)-lactamase gene was recovered by plating the gene library on to plates containing kanamycin (25 mg/L) and ceftazidime (6 mg/L). Sequencing was carried out on both strands by the dyeoxide-chain termination method with a Perkin Elmer Biosystems 377 DNA sequencer. Sequence analysis was performed using the Lasergene DNASTAR software package. Sequence alignments were performed using Clustal_W with a PAM 250 matrix.

**Results**

\(P. aeruginosa\) 81-11963A produces a VIM-type \(\beta\)-lactamase

By NCCLS criteria, \(P. aeruginosa\) 81-11963A was susceptible only to polymyxin (MIC 2 mg/L). The isolate was immediately susceptible to aztreonam (MIC 16 mg/L), amikacin (MIC, 32 mg/L) and ciprofloxacin (MIC 2 mg/L) and highly resistant (MIC > 256 mg/L) to all other common antimicrobial agents tested, including penicillins, cephalosporins and carbapenems. When the isolate was screened with the imipenem-EDTA Etest strip (AB Biodisk, Solna, Sweden), the MIC of imipenem decreased from >256 to 4 mg/L in the presence of the metal chelator, EDTA, indicating production of a zinc-dependent \(\beta\)-lactamase. To determine which type of enzyme was produced, the strain was investigated for carriage of \(bla\textsubscript{IMP}\) or \(bla\textsubscript{VIM}\) type genes. Two PCR primer pairs, one targeted to conserved regions of \(bla\textsubscript{IMP}\) type genes and the other to \(bla\textsubscript{VIM}\) type genes, were used in low stringency PCRs (annealing temperature 45°C). Metallo-\(\beta\)-lactamase ‘positive controls’ were strains of \(P. aeruginosa\) carrying \(bla\textsubscript{IMP}\) or \(bla\textsubscript{VIM}\). Using genomic DNA from \(P. aeruginosa\) 81-11963A, a PCR amplicon of the expected size was obtained using the primer pair to detect \(bla\textsubscript{VIM}\) type genes, but not with the \(bla\textsubscript{IMP}\) type primers (data not shown).

**Cloning of \(bla\textsubscript{VIM}\) and adjacent DNA from \(P. aeruginosa\) 81-11963A**

The gene encoding the metallo-\(\beta\)-lactamase was isolated from a size-fractionated genomic library, as described previously. Approximately 20 colonies were isolated and subsequent analysis determined ceftazidime MICs were >128 mg/L. In the presence of EDTA (10 mM) the ceftazidime MICs decreased from 256 to <4 mg/L, confirming that the recombinant plasmids carried a metallo-\(\beta\)-lactamase gene. \(E. coli\) carrying the recombinant plasmid gave imipenem and meropenem MICs of 1 and 0.125 mg/L, respectively. One clone, pMATWS1, containing an insert of ~5 kb was further analysed by sequencing to assess the genetic context of the \(bla\textsubscript{VIM}\) derivative. The insert of \(P. aeruginosa\) DNA in this plasmid was sequenced. The sequence has been deposited in the EMBL database under accession number AJ515707.

**Sequence analysis of the \(P. aeruginosa\) 81-11963A DNA insert in pMATWS1**

Analysis of the nucleotide sequence of the DNA from \(P. aeruginosa\) 81-11963A carried on recombinant plasmid pMATWS1 revealed the presence of \(bla\textsubscript{VIM}\) (Figure 1). Adjacent to and downstream from \(bla\textsubscript{VIM}\) was \(aacA4\), a gene that encodes AAC(6’)-Ib, which confers resistance to the aminoglycosides, kanamycin and netilmicin. Between \(aacA4\) was the 3’-CS region typical of class 1 integrons. On the other side of the \(bla\textsubscript{VIM}\) gene was \(intL\), encoding the integrase of a class 1 integron. Between \(intL\) and \(bla\textsubscript{VIM}\) was an \(attL\) site. Thus, \(bla\textsubscript{VIM}\) from \(P. aeruginosa\) 81-11963A was a component of a previously undescribed class 1 integron. While the \(aacA4\) gene on the integron was followed by a typical 59 base element (be), as previously reported, the \(bla\textsubscript{VIM}\) gene was not. Instead, there was a truncated version of only 19 base pairs (bp) (Figure 2), comprising an inverse core site and a core site separated by only 5 bp.

**Discussion**

This is the first report of a \(P. aeruginosa\) strain possessing a metallo-\(\beta\)-lactamase gene in Central Europe and illustrates the continuing dissemination of these genes throughout the continent. While strain 81-11963A was resistant to virtually all antimicrobials, the cloned gene, pMATWS1, when expressed in \(E. coli\) conferred resistance to all \(\beta\)-lactams except the carbapenems and aztreonam (data not shown). Despite this, crude cell extracts from both strain 81-11963A and \(E. coli\) (pMATWS1) demonstrated hydrolytic activity against imipenem that was fully inhibited by the presence of EDTA (20 mM) (results not shown).

The \(bla\textsubscript{VIM}\) gene from strain 81-11963A could not be transferred to a susceptible \(P. aeruginosa\) recipient, and was shown by PCR to be chromosomally encoded as judged by \(bla\textsubscript{VIM}\) amplicons obtained from genomic DNA preparations but not plasmid DNA preparations.
The blaVIM-2-containing integron from *P. aeruginosa* 81-11963A was very similar to the blaVIM-2-containing integron, In58, found in France. In58 also carries the aacA4 gene cassette, but is separated from the blaVIM-2 gene cassette by an aacC1 gene cassette. These gene cassette assortments are somewhat different from other blaVIM-2-containing integrons (Figure 1). The blaVIM-2 gene recovered from *P. aeruginosa* 81-11963A was unusual in that it is not accompanied by a full-size 5′-CS. Instead, it was followed by what appears to be a deleted 5′-CS of 19 bp. The deletion appears to have removed all but 5 bp of the sequence between the inverse core site and the core site.
of the 59 be (Figure 2). When the sequences were analysed further, it seems likely that the deleted element was the result of integrase-mediated excision. Indeed, the integron found in P. aeruginosa 81-11963A can be derived readily from In58 by two integrase-mediated gene cassette excisions. The first would remove the aacA7 gene cassette to leave the blaVIM2 gene cassette at the attI site. The second excision would remove the aacC1 gene cassette and part of the blaVIM2 gene cassette 59 be as the result of integrase matkine the 2L sequence (GTTCAG) of the blaVIM2 gene cassette 59 be for the core sequence (TTAGAT) of the 59 be, separating the blaVIM2 and aacC1 genes (Figure 2), where recombination would normally occur. Such an event would fuse the first 13 bp of the blaVIM2 gene cassette 59 be, GCATAACATGAAG, to the terminal 6 bp, TTAGGC, of the core site of the 59 be separating the aacC1 and aacA4 genes, in the process creating the 19 bp hybrid element found (Figure 2). Database searches for other examples of shortened 59 be revealed two

References