Novel Variant (*bla*_{VIM-4}) of the Metallo- β -Lactamase Gene *bla*_{VIM-1} in a Clinical Strain of *Pseudomonas aeruginosa*

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Received 11 April 2002/Returned for modification 16 May 2002/Accepted 11 September 2002

A *Pseudomonas aeruginosa* isolate highly resistant to carbapenems was collected from a patient with post-surgical cerebrospinal infection in Greece. The isolate carried a class 1 integron that contained as a sole cassette the gene *bla*_{VIM-4}, a novel variant of *bla*_{VIM-1}, with one nucleotide difference resulting in a Ser-to-Arg change at amino acid position 175 of the VIM-1 enzyme. This is the first detection of a VIM-1 variant after its appearance in Italy.

There is an increasing number of studies reporting on the emergence of *Pseudomonas aeruginosa* strains that produce acquired class B metallo- β -lactamases (MBLs), which invariably hydrolyze carbapenems. So far, two distinct MBL types, IMP (11) and VIM (2), have been described. Although these β -lactamase types share less than 40% amino acid sequence identity, they exhibit comparable kinetic properties, inactivating virtually all β -lactams except monobactams (7). Additionally, both *bla*_{IMP}- and *bla*_{VIM}-type genes are carried as gene cassettes by class 1 integrons. Of the VIM MBLs, VIM-1 was the first to be identified, but up to now it had been described only for *P. aeruginosa* and *Achromobacter xylosoxidans* from Italy (1, 4, 16). In contrast, VIM-2 enzymes have been detected in clinical *Pseudomonas* isolates in several Mediterranean countries and the Far East (5, 8, 12, 14, 15, 19). A variant of VIM-2 differing by two amino acids, VIM-3, was also detected in clinical isolates of *P. aeruginosa* from Taiwan (19). In this study we report on the identification of *bla*_{VIM-4}, a novel variant of the VIM-1 MBL gene, found in a clinical strain of *P. aeruginosa*.

The carbapenem-resistant *P. aeruginosa* strain P1936 was studied. *P. aeruginosa* ATCC 27853 was used as a control strain. *P. aeruginosa* P1936 was isolated on 23 April 2001 in the University Hospital of Thessaly, Larissa, Greece. The strain was repeatedly recovered as a sole isolate from the ventriculo-peritoneal catheter and the cerebrospinal fluid of a 25-year-old man 20 days after a neurosurgical operation for the management of a posttraumatic hydrocephalus. The patient was readmitted to the hospital 18 days after the operation with high fever, respiratory distress, and clinical signs of meningitis. He also had simultaneous positive blood cultures that yielded *Acinetobacter baumannii* and *Enterococcus faecium* but not *P. aeruginosa*. Prior to the isolation of these microorganisms, the patient had been treated with multiple antibiotic courses that included ceftazidime, imipenem, ciprofloxacin, clindamy-

cin, and metronidazole. Based on the results of the susceptibility tests, the patient was treated with imipenem, aztreonam, amikacin, and vancomycin, and the catheter was removed. The patient's clinical condition gradually improved, and the cultures became negative.

The susceptibility of *P. aeruginosa* P1936 to several antimicrobial agents including β -lactams, tobramycin, gentamicin, amikacin, netilmicin, and ciprofloxacin was assessed by disk diffusion (10). The MICs of ticarcillin-clavulanic acid, piperacillin-tazobactam, ceftazidime, aztreonam, imipenem, and meropenem were determined by an agar dilution method (9).

Extraction of plasmid DNA was carried out by using the Qiagen Plasmid kit (Hilden, Germany) according to the manufacturer's instructions. These extracts were used to transform *Escherichia coli* DH5 α cells with transformant selection on Mueller-Hinton agar containing ampicillin (50 μ g/ml).

*bla*_{VIM}-type genes were detected by PCR with a pair of consensus primers under amplification conditions as described previously (18). Primers specific for *bla*_{VIM-1} and *bla*_{VIM-2} were also used (19). PCR assays combining primers specific for conserved 5'-CS and 3'-CS sequences (6) with the *bla*_{VIM}-specific ones were also performed to investigate the possible association of the MBL gene with a class 1 integron.

Nucleotide sequencing of both strands of the PCR products derived using the 5'-CS and *bla*_{VIM-1} (reverse) primers (19) was done by using an ABI Prism 377 DNA sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, Calif.).

β -Lactamase extracts from *P. aeruginosa* P1936 and the control strains were obtained by ultrasonic treatment of cell suspensions and clarified by centrifugation. Protein concentrations were determined with a protein assay kit (Bio-Rad, Richmond, Calif.). Carbapenemase activity was estimated by spectrophotometry as described previously (4). The maximum hydrolysis rate of imipenem was monitored at 299 nm and expressed as units of activity (1 unit is defined as 1 pmol of imipenem hydrolyzed per min per μ g of protein). Isoelectric focusing (IEF) was performed in polyacrylamide gels containing ampholytes (pH range, 3.5 to 9.5). β -Lactamase activity was visualized with nitrocefin.

P. aeruginosa P1936 was examined in the context of the

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regular screening for MBL producers among carbapenem-resistant nonfermenters (mostly *Pseudomonas* and *Acinetobacter* spp.) that have sporadically appeared in the University Hospital of Thessaly since 1999. The strain was highly resistant to all β -lactam antibiotics except aztreonam, which retained a moderate activity (MIC, 16 μ g/ml). It was also resistant to all four aminoglycosides tested and to ciprofloxacin. Transformation experiments did not yield any β -lactam-resistant *Escherichia coli* clone.

Total DNA from P1936 was positive in the PCR assay using the consensus *bla*_{VIM} primers. PCR products of similar size (261 bp) were also detected with 12 other carbapenem-resistant *P. aeruginosa* strains isolated during the time period from September 1999 to August 2001 in this hospital (data not shown). While the latter strains were all found to carry *bla*_{VIM-2}-type genes, *P. aeruginosa* P1936 was positive for *bla*_{VIM-1}, producing in the respective PCR assay an amplicon of the expected size (920 bp). PCRs using the 5'-CS and *bla*_{VIM-1} (reverse) primers as well as the 3'-CS and *bla*_{VIM-1} (forward) primers yielded products of similar sizes (approximately 950 bp), indicating that *bla*_{VIM-1} was the sole gene cassette in the variable region of this integron.

The DNA sequence of the PCR amplicon encompassed by the 5'-CS and *bla*_{VIM-1} (reverse) oligonucleotides showed an 870-bp segment that corresponded to a fraction of the VIM-1 integron (from nucleotide 1185 to nucleotide 2054; GenBank accession no. Y18050) originally described by Lauretti et al. (4). The sequence included a part of the 5' conserved segment followed by a *bla*_{VIM-1}-type gene that possessed a C instead of an A at nucleotide position 1864 (numbering according to Y18050). This transversion resulted in a Ser-to-Arg change at position 175 (numbering according to reference 4) of the VIM-1 MBL. It is notable that position 175 is also occupied by Arg in VIM-2 and VIM-3 enzymes. Residue 175 (position 228 in the recently proposed standard numbering scheme for class B β -lactamases [3]) is not among those considered significant for β -lactam hydrolysis by VIM and IMP MBLs (3). This novel *bla*_{VIM-1} variant was designated *bla*_{VIM-4}.

Photometric assays showed that crude β -lactamase preparations from *P. aeruginosa* P1936 exhibited an activity against imipenem that was comparable to that observed with extracts containing VIM-2 (data not shown). It was therefore assumed that *bla*_{VIM-4} was expressed and was, at least partly, responsible for the high-level resistance of *P. aeruginosa* P1936 to carbapenems. The enzyme, however, could not be detected in the IEF experiments. Prolonged incubation of the IEF gels with nitrocefin indicated a weak β -lactamase activity at a pI of 8.8, which probably represented the chromosomal cephalosporinase. Other major β -lactamase bands were not observed.

Initially, IMP MBLs were restricted to the Far East, while VIM types were found exclusively in southern Europe (7, 13, 17). However, during the last few years, VIM-type genes carried by a variety of integron structures have been found in various gram-negative rods in the Far East (5, 19). VIM-4- and VIM-1-encoding genes differ by only one nucleotide and should be considered alleles derived from a common ancestor. On the other hand, *bla*_{VIM-4} was, most likely, the sole gene in the variable region of the detected class 1 integron while the *bla*_{VIM-1} integrons found in sporadic isolates in Italy included additional resistance gene cassettes (4, 16), indicating different

phylogenies. Given also that there was not any apparent epidemiological association of the case described here with those in Italy, it can be assumed that the *bla*_{VIM-4}-encoding integron emerged independently.

The results of the present study, taken together with previous findings (18), indicate that VIM-producing *P. aeruginosa* strains have already been established in Greek hospitals. Epidemiological surveillance in the major tertiary care institutions, restriction of carbapenem usage, and adjustment of the infection control measures should be considered.

Nucleotide sequence accession number. The GenBank accession no. of the nucleotide sequence reported here is AY135661.

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