MAJOR ARTICLES

Hospital Outbreak of Carbapenem-Resistant *Pseudomonas aeruginosa* Producing VIM-1, a Novel Transferable Metallo-β-Lactamase

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A total of 8 *Pseudomonas aeruginosa* isolates was collected from 7 different patients in different wards of the University Hospital of Verona, Italy, from February 1997 to February 1998. The high level of resistance to carbapenems (imipenem minimum inhibitory concentration was always >128 μg/mL) and other broad-spectrum β-lactams and the rate of imipenem hydrolysis and its inhibition by ethylenediamine–tetra-acetic acid were all suggestive of production of a carbapenem-hydrolyzing metallo-β-lactamase. A specific DNA probe derived from the recently cloned bla_{VIM-1} gene hybridized to all the isolates. A genomic DNA fingerprinting profile revealed clonal relatedness for 7 of 8 isolates. A description of this hospital outbreak is reported, the occurrence of which confirms that proliferation of metallo-β-lactamase–producing strains multiply resistant to β-lactams is already a reality outside Japan. These findings emphasize the need for early recognition of similar isolates.

Pseudomonas aeruginosa are among the bacteria most commonly isolated from nosocomial infections. They exhibit intrinsic resistance to several antimicrobial agents, including most β-lactams, and they often carry acquired resistance determinants that further reduce their spectrum of susceptibility [1–5]. Carbapenems have the broadest antipseudomonal spectra of all β-lactams and play a fundamental role in the treatment of *P. aeruginosa* infections. Resistance to carbapenems is often associated with the loss of the 46–48-kDa outer-membrane protein D2 [6], which has been renamed OprD. A decrease in OprD expression regularly accompanies overexpression of the multidrug efflux system MexE-MexF-OprN, thus accounting for the carbapenem resistance observed in those strains [7]. In OprD-deficient strains, derepressed production of chromosomal β-lactamases significantly contributes to imipenem resistance [2], but of greater concern are the increasingly frequent reports of "carbapenemases," that is, enzymes that are able to hydrolyze carbapenems with fast kinetics [8].

Clinical Infectious Diseases 2000;31:1119–25

Until recently, the occurrence of *P. aeruginosa* isolates producing acquired carbapenemases had been reported only in the Far East, where the mobile bla_{IMP} determinant encoding the broad-spectrum metallo-β-lactamase IMP-1 has been spreading among *Enterobacteriaceae, P. aeruginosa,* and a number of other nonfastidious gram-negative nonfermenters since the late 1980s [9–10]. Recently, however, carbapenemase-producing *P. aeruginosa* isolates have also been detected in the United Kingdom, Italy, and Portugal [11–13], suggesting that this fearsome mechanism of resistance could soon become a global problem.

The carbapenemase-producing *P. aeruginosa* isolate from Italy (VR-143/97) was obtained from the Verona University Hospital (northern Italy) and was found to produce a novel metallo-β-lactamase, which was named VIM-1. Like IMP-1, it exhibits a very broad substrate specificity and is encoded by a determinant (bla_{VIM}) carried on a mobile gene cassette inserted in an integron [11]. Following isolation of VR-143/97, systematic analysis of *P. aeruginosa* isolates was carried out at the University Hospital of Verona over a 1-year period in order to investigate the overall prevalence of carbapenem-resistant isolates from nonurinary clinical specimens and the presence of bla_{IMP}, bla_{VIM}, or other acquired carbapenemase determinants in the resistant strains. That survey detected additional *P. aeruginosa* isolates producing VIM-1 or closely related variants from the same hospital [14]. We carried out a retrospective analysis of the cases from which the metallo-β-lactamase–producing *P. aeruginosa* were isolated, including evaluation of various clinical and epidemiological parameters and of some relevant features of the microbial isolates.

Materials and Methods

Bacterial strains and screening procedures. A total of 541 clinical isolates of *P. aeruginosa* were collected from nonurinary specimens at the University Hospital of Verona, Italy, from February 1997 to February 1998. A number of these isolates were selected for further experiments on the basis of the following criteria, the simultaneous presence of which could be suggestive of production of a carbapenem-hydrolyzing metalloenzyme: resistance to imipenem; resistance to other broad-spectrum β-lactams, such as ceftazidime; and susceptibility to or an intermediate phenotype to aztreonam.

Antibiotics. Working solutions were prepared on the day of use from laboratory standard powders of all compounds as specified by the manufacturers. Meropenem was provided by Zeneca (Milan, Italy). Cilastatin-free imipenem was provided by Merck Sharp & Dohme (Rome, Italy). All other antibiotics were from commercial sources.

Susceptibility tests. Disk susceptibility tests were performed and interpreted according to the latest National Committee for Clinical Laboratory Standards (NCCLS) documents [15–16]. Dilution antimicrobial susceptibility tests were performed by serial 2-fold dilution in Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, MD) and interpreted as described in the latest NCCLS documents [16–17].

β-Lactamase assays. The hydrolysis rates of 100 μM antibiotic by sonic extracts of cells were obtained as previously described [18] with a Beckman DU-7 ultraviolet spectrophotometer (Palo Alto, CA), with readings recorded at 10-s intervals for 5 min, operating at the wavelength at which the difference between the extinction values of the hydrolyzed and nonhydrolyzed molecule was maximum. The exact wavelengths used were as follows: ceftazidime, 260 nm; and imipenem and meropenem, 299 nm. The millimolar differential absorbance value for all the β-lactams tested was 9.0 mM/cm for imipenem and ceftazidime and 6.5 mM/cm for meropenem, at the respective wavelengths. Inhibition of enzymatic activity by EDTA was determined by measuring the residual carbapenemase activity after incubation of the crude extract for 20 min at 25°C in the presence of 2 mM of EDTA. A control without EDTA was always run in parallel. Reactivation by Zn^{2+} was determined by measuring the carbapenemase activity after incubation of the EDTA-treated enzyme preparation for 20 min at 25°C in the presence of 2 mM Zn^{2+}, as described previously [11]. Controls for the effect of Zn^{2+} alone on enzyme activity and substrate stability were also included.

Isoelectric focusing analysis. Gel isoelectric focusing was performed in 5% polyacrylamide gels containing ampholytes (pH range, 5.5–9.5) with a Multiphor II apparatus (Pharmacia-LKB Biotechnology, Uppsala, Sweden). The gels were focused at 4°C and 25 W for 180 min. The isolectric point (pI) value was determined by focusing 5 μg of each purified enzyme. β-Lactamases were detected by overlaying the gel with 0.5 $\mu mol/mL$ nitrocefin in 0.1 mmol/mL phosphate buffer, pH 7.0, applied as a nitrocefin-soaked filter paper. Carbapenem hydrolyzing activity was assayed by overlaying the gel with imipenem-containing nutrient agar and swabbing a susceptible indicator organism across the agar [19].

Characterization of outer-membrane proteins. Outer-membranes were prepared by a rapid procedure [6, 18] yielding appreciable degrees of purification. Cells were grown in Luria-Bertani broth up to the late exponential phase of growth, harvested by centrifugation, washed once with 20 mM HEPES-NaOH buffer (pH 7.4) containing 1 mM phenyl-methylsulfonal fluoride, and resuspended in the same buffer. The suspension was sonicated with the above-mentioned ultrasonic disrupter by 4 30-s pulses of sonication with an intervening 30 s in ice. The unbroken cells were removed by centrifuging at 1500 *g* for 15 min, and membranes were pelleted by centrifugation of the supernatant at 19,000 *g* for 30 min. Finally, pellets were resuspended in 1 mL of HEPES-NaOH buffer 20 mM, pH 7.4, and stored at −80°C. The protein content of the samples was determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Outer-membrane proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) essentially as described by Laemmli [20]. Proteins were detected by Coomassie blue staining.

Pulsed-field gel electrophoresis (PFGE). Chromosomal DNA was prepared from each strain for analysis by PFGE by the method described by Smith and Cantor [21] as modified by Ichiyama et al. [22] and was digested with *Xba*I for 24 h at 37°C. DNA fragments were separated with a CHEF DR III apparatus (Bio-Rad). Half-strength Tris borate–EDTA running buffer was used, and the electrophoresis was run at 14°C for 23 h at 200 V. Pulse time was linearly ramped within an initial forward time of 5 s and a final forward time of 35 s. Interpretation of DNA restriction patterns generated by PFGE was according to the guidelines proposed by Tenover et al. [23].

Colony blot hybridization. Colony blot hybridization was performed with cells grown directly on sterile nitrocellulose filters (Schleicher & Schuell, Keene, NH) layered on Mueller-Hinton agar. Cell lysis, prehybridization, and hybridization were carried out essentially as described elsewhere [24]. DNA probes were labeled with ^{32}P by the random priming technique with a commercial kit (Boehringer, Mannheim, Germany). The bla_{IMP}-specific probe was a 0.5-kb *Hin*dIII fragment internal to the bla_{IMP} gene [25]. The bla_{VIM}-specific probe was a PCR-generated amplicon containing the entire bla_{VIM} coding sequence from *P. aeruginosa* VR-143/97 [11].

Results

Origins and screening of the isolates. Of the 541 strains collected from nonurinary specimens over the period from February 1997 to February 1998, as many as 244 strains (45.1%) were isolated from lower respiratory tracts, whereas decidedly lower percentages of strains came from surgical wounds (11.6%), pharyngeal swabs of patients with hematological disorders (7.4%), and blood cultures (6.8%). Of the 541 isolates, 83 (15.3%) demonstrated imipenem resistance (MIC, ⩾16 μg/mL). Of these, 62 (11.4% of all isolates) exhibited resistance also to ceftazidime (MIC, ⩾32 μg/mL), and 35 of the latter group (6.5% of all isolates) also proved susceptible or intermediate to aztreonam (MIC, <32 μg/mL). These 35 isolates were selected for further experiments.

Analysis of carbapenemase production. In 7 of 35 strains selected in the previous step (the VIM-1–producing index strain VR-143/97 [11] was one of them), the crude cell-free extracts

Table 1.   Clinical associations of *Pseudomonas aeruginosa* strains carrying the metallo-β-lactamase gene.

Strain	Patient	Ward	Source	Collection date	Previous therapy with	Outcome of hospitalization[a]
VR-143/97	NG	ICU	Pus	1 February 1997	Imi	Death
VR-146/97	GB	ICU	Bronchial aspirate	25 March 1997	Imi	Discharge
VR-155/97	SL	Surgery	Bile	27 March 1997	Czid	Death
VR-158/97	RS	ICU	Bronchial aspirate	9 July 1997	Ctri	Death
VR-170/97	RS	ICU	Bronchial aspirate	31 July 1997	Ctri	Death
VR-174/97	CE	ICU	Blood	11 September 1997	Imi, Czid	Death
VR-186/98	BC	ICU	Bronchial aspirate	5 February 1998	Amox/Clv	Death
VR-193/98	CF	Hematology	Pharyngeal swab	20 February 1998	Czid	Discharge

NOTE.   Amox/Clv, amoxicillin/clavulanate; Ctri, ceftriaxone; Czid, ceftazidime; ICU, intensive care unit; Imi, imipenem.

[a] Because of the retrospective nature of the analysis and because of the critical status of the patients, a definite correlation between *Pseudomonas* infection and death was in any case difficult to establish.

showed fast hydrolysis of both imipenem and meropenem, confirming specific carbapenem-hydrolyzing activity, and also of the third-generation cephalosporin ceftazidime (data not shown). Carbapenemase activity was inhibited by treatment with EDTA and could be fully restored following addition of Zn^{2+} to the EDTA-treated sample (see also Lauretti et al. [11]). On the basis of these data, all 7 isolates were judged to be eligible for further analysis. Because all these isolates lacked any inhibition zone in the Kirby-Bauer susceptibility test, a further (eighth) isolate sharing the same feature, namely VR-155/97, which had been originally disregarded as aztreonam resistant, was also examined for metallo-β-lactamase production. The assay was also positive with this isolate, which was therefore considered for further analysis along with the other 7 strains.

After development with nitrocefin, analytical isoelectric focusing of the crude extracts of the 8 isolates revealed multiple bands of β-lactamase activity in all of them, including 1 large band plus minor ones at alkaline pH values (~7.5–8.8) and 1 band at acidic pH value (~5.3) (data not shown). The alkaline pI bands were probably contributed by the resident AmpC-like cephalosporinase of *P. aeruginosa*, whereas the acidic one (whose value was similar to that previously reported for VIM-1 [11]) was contributed by the carbapenemase, as demonstrated by its detection by means of the gel overlay procedure with imipenem.

A bla_{VIM}-specific probe derived from the bla_{VIM} gene cloned from VR-143/97 yielded a strong hybridization signal with all the carbapenemase-producing isolates in colony hybridization experiments, with the intensity of the signal similar to that obtained with VR-143/97. None of the above isolates was recognized by a bla_{IMP}-specific probe in a similar hybridization assay.

Clinical associations of the selected isolates.   The 8 isolates under investigation came from 7 different inpatients. Most of them had been isolated from patients given various combinations of antimicrobial agents, including broad-spectrum cephems (ceftriaxone or ceftazidime), imipenem, or both, before identification. The clinical associations of the 8 *P. aeruginosa*

isolates carrying the metallo-β-lactamase gene are summarized in table 1, and a brief analytical description of each of them is reported below.

Patient NG.   Patient NG, the index case, was a 37-year-old man with respiratory failure of neurogenic origin (transverse myelitis). He was transferred from the neurology department to the intensive care unit (ICU) in January 1997 and mechanically ventilated. Therapy with imipenem (0.5 g given iv every 8 h) was initiated on day 10 after admission and was never discontinued. *P. aeruginosa* (isolate VR-143/97) was isolated from the pus of a surgical wound on day 15 of imipenem therapy (day 25 after admission to the ICU). Death occurred on day 60 after admission to the ICU.

Patient GB.   Patient GB was a 71-year-old man admitted to the coronary unit for severe heart failure in March 1997. He underwent surgery on day 7 after admission for an aortic prosthesis implant. *Serratia marcescens* was isolated from bronchial aspirate on day 10 after surgery. The patient was transferred to the ICU on day 15 after surgery because of postoperative heart failure and shock. Therapy with imipenem (0.5 g iv every 8 h) was started on the day of admission and was never discontinued. *P. aeruginosa* (isolate VR-146/97) was isolated from bronchial aspirate on day 18 of imipenem therapy, exactly 1 day after the patient had been discharged from the ICU and transferred to the geriatrics department. This patient was ultimately discharged from the hospital after a further 20 days.

Patient SL.   Patient SL was a 58-year-old man who underwent surgery in January 1997 because he had colon cancer with hepatic metastasis. During hospitalization, he was frequently admitted to the ICU for placement of central venous catheters. Therapy consisted of ceftazidime (2 g iv every 8 h) for 20 days and imipenem (0.5 g iv every 8 h) for a further 10 days. *P. aeruginosa* (isolate VR-155/97) was isolated from bile on day 10 of ceftazidime therapy. The patient was transferred to the department of medicine in May 1997, but he died 30 days after being admitted.

Patient RS.   Patient RS was a 5-year-old boy with leukoencephalopathy admitted to the ICU in May 1997 for severe neurological deficits and generalized convulsions. On day 8 af-

Table 2. Susceptibility profiles of the *Pseudomonas aeruginosa* isolates carrying the bla_{VIM} metallo-β-lactamase gene.

Isolate	MIC of antimicrobial agent, μg/mL											
	Imi	Mero	Ctri	Czid	CEP	Atm	Pip/Taz	Tic/Clv	Gm	Amik	Tm	Cpfx
VR-143/97	>128	128	>128	>128	>128	16	>128	>128	64	32	32	>128
VR-146/97	>128	>128	>128	>128	>128	8	>128	>128	>128	>128	>128	>128
VR-155/97	>128	>128	>128	>128	>128	32	>128	>128	>128	>128	>128	>128
VR-158/97	>128	128	>128	>128	>128	8	>128	>128	32	32	32	>128
VR-170/97	>128	128	>128	>128	>128	16	>128	>128	>128	64	64	>128
VR-174/97	>128	>128	>128	>128	>128	8	>128	>128	>128	64	64	>128
VR-186/98	>128	>128	>128	>128	>128	4	>128	>128	>128	64	64	>128
VR-193/98	>128	>128	>128	>128	>128	4	>128	>128	128	32	32	>128

NOTE. Amik, amikacin; Atm, aztreonam; CEP, cefepime; Cpfx, ciprofloxacin; Ctri, ceftriaxone; Czid, ceftazidime; Gm, gentamicin; Imi, imipenem; Mero, meropenem; Pip/Taz, piperacillin/tazobactam; Tic/Clv, ticarcillin/clavulanate; Tm, tobramycin.

ter admission to the ICU, therapy was initiated with ceftriaxone (1 g iv every 12 h) for 30 days, owing to the onset of fever (38°C–39°C). *Citrobacter freundii* was isolated from bronchial aspirate on day 28 of ceftriaxone therapy (day 36 after admission). *P. aeruginosa* (isolates VR-158/97 and VR-170/97) were isolated from bronchial aspirate on days 60 and 80, respectively, after admission. Death occurred on day 90 after admission.

Patient CE. Patient CE was a 71-year-old woman who underwent surgery in May 1997 for acute necrotic pancreatitis. During the following 2 months, several microorganisms (namely *Klebsiella pneumoniae*, imipenem-susceptible *P. aeruginosa*, *Bacteroides fragilis*, *Stenotrophomonas maltophilia*, and *Acinetobacter baumannii*) were isolated from blood cultures and bronchial aspirates, in spite of consecutive therapies with imipenem (0.5 g iv every 8 h) and ceftazidime (2 g iv every 8 h). After frequent admissions to the ICU (day hospital) for placement of central venous catheters, the patient was finally transferred to the ICU in late July 1997 because of severe shock. On day 20 after the admission to the ICU, therapy with imipenem (0.5 g iv every 8 h) was initiated owing to onset of fever (38.8°C) and was continued for 6 days. *P. aeruginosa* (isolate VR-174/97) was isolated from blood culture on day 18 after discontinuation of the therapy (day 44 after admission). Death occurred on day 50 after admission to the ICU.

Patient BC. Patient BC was a 61-year-old man admitted to the ICU in late January 1998 because he suffered a cerebral vascular accident, which was followed by severe cerebral ischemia and coma. Therapy with amoxicillin plus clavulanic acid (1.2 g iv every 8 h) was started on the day of admission and was never discontinued. *P. aeruginosa* (isolate VR-186/98) was isolated from bronchial aspirate on day 6 after admission, and the patient died on the same day.

Patient CF. Patient CF was a 34-year-old man with acute leukemia admitted to the hematology department in February 1998 in order to receive bone marrow transplantation. On day 8 after transplantation, therapy was initiated with ceftazidime (2 g iv every 8 h) because of onset of fever (>38°C) and continued for 7 days. *P. aeruginosa* (isolate VR-193/98) was isolated

from a pharyngeal swab on day 2 of ceftazidime therapy; all other specimens were negative. The transplantation proved successful, and the patient was discharged 30 days after admission.

Susceptibility testing. The results of susceptibility testing of 12 antimicrobial agents with the 8 bla_{VIM}-positive isolates are shown in table 2. All isolates demonstrated high-level resistance to carbapenems, to all broad-spectrum cephems including ceftazidime (MIC, ≥128 μg/mL), and to inhibitor-protected penicillins; MICs of aztreonam appeared to be lower and, apart from isolate VR-155/97, below the breakpoint for resistance (32 μg/mL). All isolates also demonstrated high-level resistance to non–β-lactam agents, including the quinolone ciprofloxacin and the aminoglycosides gentamicin and tobramycin; amikacin had similar MIC values that, however, would allow 4 strains to be classified as "intermediate" according to the NCCLS breakpoints (MIC, 32 μg/mL).

Outer-membrane protein analysis. Analysis of SDS-PAGE gels of outer-membrane proteins revealed that all isolates lacked one outer-membrane protein that was present in a susceptible counterpart (figure 1). By comparing these SDS-PAGE patterns with those shown by a reference pair of imipenem-susceptible and imipenem-resistant strains [6, 26], it was possible to confirm the virtual absence of a protein with an apparent molecular weight very close to OprD.

Clonal analysis of the bla_{VIM}-*positive isolates.* The chromosomal DNA macrorestriction patterns of the 8 bla_{VIM}-positive isolates, obtained after digestion with *Xba*I, were analyzed by PFGE. The restriction patterns were found to be identical in 4 isolates and different for 1 band in 3 isolates, suggesting that all these isolates were clonally related. However, the restriction profile of VR-193/98 was different for >4 bands from the other profiles, suggesting that it was not clonally related to the others (figure 2).

Discussion

The spread of metallo-β-lactamases capable of hydrolyzing carbapenems in *P. aeruginosa* and other major gram-negative
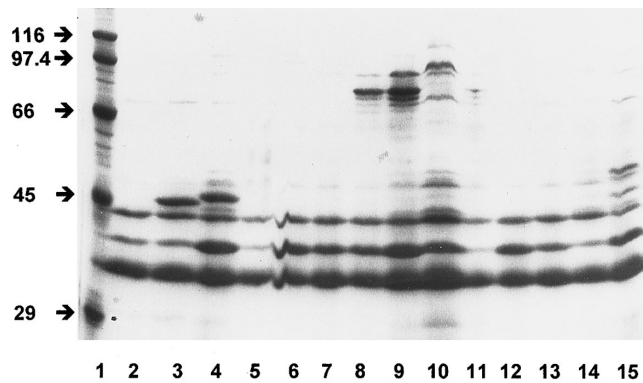
Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of outer-membrane proteins from clinical isolates of *Pseudomonas aeruginosa*. *Lane 1,* Molecular weight markers: β-galactosidase (116,000); phosphorylase B (97,400); bovine albumin (66,000), egg albumin (45,000), and carbonic anhydrase (29,000). *Lane 2,* Imipenem-resistant OprD-deficient strain 3B [27]. *Lane 3,* Imipenem-susceptible strain 3C [27]. *Lane 4,* Imipenem-susceptible strain VR-188/98. *Lane 5,* VR-143/97. *Lanes 6* and *7,* VR-146/97 (in duplicate). *Lanes 8* and *9,* VR-155/97 (in duplicate). *Lane 10,* VR-158/97. *Lane 11,* VR-170/97. *Lanes 12* and *13,* VR-174/97 (in duplicate). *Lane 14,* VR-186/98. *Lane 15,* VR-193/98.

pathogens has been a worrying problem in Japanese hospitals since the early 1990s [9], and recent reports suggest that a similar phenomenon is now also emerging in Europe [12–13]. In Italy, the first outbreak of metallo-β-lactamase–producing *P. aeruginosa* was detected in the University Hospital of Verona in 1997–1998 and was caused by strains that produced a new broad-spectrum class B enzyme, named VIM-1, encoded by the mobile bla_{VIM} gene [7, 11]. We carried out an analysis of that outbreak by evaluating various clinical, epidemiological, and microbiological parameters. Although the retrospective nature of the survey permitted evaluation only of a number of clinical and epidemiological variables (ward, underlying diseases and overall clinical history, and antimicrobial chemotherapy), analysis of the various parameters yielded the following considerations.

Although it was not possible to trace the exact origin of the outbreak and its subsequent spread, a common denominator among most patients (6 of 7) consisted in ≥1 admissions to the ICU before isolation of the bla_{VIM}-positive *P. aeruginosa*, suggesting that in most cases the microorganism had probably been acquired in the ICU. Clonal analysis data supported this view, indicating that the 7 isolates of patients that had ≥1 ICU admissions in common were clonally related to each other while being unrelated to the single isolate (VR-193/98) of the patient that did not report ICU admission. The ICU consists of a single room where infected patients are not isolated from other patients, an arrangement that may have had a bearing on the diffusion of the most prevalent bla_{VIM}-positive clone. Appro-

priate handwashing, which is crucial to avoid transferring multiresistant strains in ICUs, was usually assessed and probably contributed to limiting the diffusion of the bla_{VIM}-positive strain in such an epidemiological setting.

The clinical significance of the isolates was difficult to assess. One of them (VR-193/98, isolated from a pharyngeal swab) was clearly a colonizer. In the 6 remaining patients, a role in infection was reasonably clear for VR-143/97 (isolated from purulent material from a surgical wound), VR-155/97 (isolated from bile), and VR-174/97 (isolated from blood), whereas it remained more doubtful for VR-146/97, VR-158/97 + VR-170/97, and VR-186/98, because the source was bronchial and the isolates could have been mere colonizers. Although 5 patients died, it was difficult to establish the causes of death with certainty (owing to the critical conditions of the patients), and, at least in some cases, death occurred several days after isolation of multiresistant *P. aeruginosa*. In any case, the presence of *P. aeruginosa* carrying a similar metallo-β-lactamase determinant in a nosocomial setting is a most worrying development from the epidemiological standpoint, because it could act as a reservoir for further spread of the resistance gene.

The diffusion of metallo-β-lactamases is highly disturbing because, contrary to what occurred when permeability-related carbapenem resistance first emerged, the isolates bearing these enzymes usually do not remain susceptible to most broad-spectrum β-lactams [9–10, 13, 26]. The fact that the bla_{VIM} determinant was found to be carried on a mobile genetic element of the gene cassette type [11] could facilitate its rapid spread among major gram-negative pathogens. In the index strain (VR-143/97), the bla_{VIM} gene was apparently carried on the chromosome and was not transferable by conjugation to *Es-*
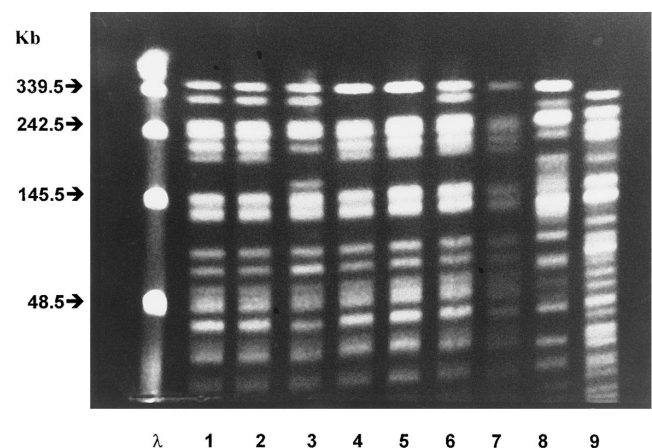

Figure 2. Pulsed-field gel electrophoresis of *Xba*I-cleaved genomic DNA of *Pseudomonas aeruginosa* clinical isolates. λ, λ Ladder DNA marker (DNA sizes indicated). *Lane 1,* VR-143/97. *Lane 2,* VR-146/97. *Lane 3,* VR-155/97. *Lane 4,* VR-158/97. *Lane 5,* VR-170/97. *Lane 6,* VR-174/97. *Lane 7,* VR-186/98. *Lane 8,* VR-193/98. *Lane 9,* VR-148/98 (control strain, not related to the outbreak).

cherichia coli [11]. It would be interesting to investigate whether a similar genetic background is also found in the other bla_{VIM}-positive isolates and to perform transfer experiments with them. In addition, because detection of the bla_{VIM} determinant was carried out by means of filter hybridization, it would also be interesting to sequence the bla_{VIM} determinants of the various isolates, and especially that of the genetically unrelated strain, to ascertain the existence of allelic variants of this resistance determinant.

Analysis of the antimicrobial chemotherapy received by the patients before isolation of the bla_{VIM}-positive *P. aeruginosa* revealed that only 3 of the patients had received therapy with imipenem, whereas the others had been given noncarbapenem β-lactams, including expanded-spectrum cephalosporins or, in one case, amoxicillin/clavulanate. Similar findings were also reported in the Japanese multifocal epidemics of bla_{IMP}-positive gram-negative rods, where previous use of carbapenems could be confirmed for only 15% of the patients, whereas 38.8% of patients were administered cephems before the isolation of bla_{IMP}-positive isolates and such strains were also isolated from patients who had not been administered antibiotics, suggesting that metallo-enzyme–bearing *P. aeruginosa* can spread as hospital infections without the use of antibiotics [26]. It should be recalled that also on the occasion of the emergence of OprD-related resistance, 1 of 3 imipenem-resistant strains was not isolated during therapy with imipenem [27].

All the bla_{VIM}-positive *P. aeruginosa* isolates in this outbreak exhibited high-level resistance not only to carbapenems and most other β-lactams but also to fluoroquinolones and several aminoglycosides. The cross-resistance to unrelated classes of antibiotics indicates the simultaneous presence of additional resistance determinants such as genes for aminoglycoside-modifying enzymes, mutations that upregulate efflux systems, or both, which are common among nosocomial isolates of *P. aeruginosa*. The different gentamicin MICs for VR-158/19 (32 μg/mL) and VR-170/97 (>128 μg/mL), which were isolated from the same patient with a 20-day difference in isolation, were probably related to the acquisition of a novel aminoglycoside resistance determinant by the latter strain, in spite of the fact that no antimicrobial chemotherapy was administered between the 2 isolations.

It is worth noting that all the bla_{VIM}-positive *P. aeruginosa* lacked the outer-membrane protein OprD and that the absence of this channel results in the acquisition of imipenem resistance [6]. Thus, the simultaneous presence of at least 2 resistance mechanisms in all isolates prevented us from assessing whether the acquisition of this novel metallo-β-lactamase gene alone is sufficient to confer high-level resistance on carbapenems or whether the simultaneous presence of an outer-membrane permeability modification is necessary for expression of high-level carbapenem resistance, as reported with IMP-1 [9]. The latter case would imply the potential existence, among metallo-β-lactamase producers, of low-level resistant strains or even of

strains that, although exhibiting a reduced carbapenem susceptibility, are still classified as "susceptible" according to the interpretative breakpoints and would be hard to detect in routine susceptibility tests. Similar strains would undoubtedly have been missed because of the protocol of our study and its requirement of imipenem resistance as a preliminary screening criterion.

During the screening for metallo-β-lactamase producers, 28 isolates were found that were resistant to imipenem and ceftazidime, that were susceptible or intermediate to aztreonam, and that did not produce any detectable carbapenemase activity. A similar phenotype could reflect the contribution of different resistance mechanisms, such as a combination of permeability defects and active efflux. These isolates undoubtedly deserve further investigation to ascertain the molecular basis of their resistance phenotype.

Acknowledgments

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