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# Nosocomial Outbreak Caused by Multidrug-Resistant *Pseudomonas aeruginosa* Producing IMP-13 Metallo-β-Lactamase

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**An outbreak of *Pseudomonas aeruginosa* showing a multidrug-resistant (MDR) phenotype (including carbapenems, ceftazidime, cefepime, gentamicin, tobramycin, and fluoroquinolones) was observed, during a 5-month period, in a general intensive care unit of a large tertiary care and clinical research hospital in southern Italy. The outbreak involved 15 patients, with a total of 87 isolates, mostly from lower respiratory tract specimens. Analysis of isolates involved in the outbreak revealed production of metallo-β-lactamase (MBL) activity, and genotyping by pulsed-field gel electrophoresis of genomic DNA digested by SpeI revealed clonal relatedness among isolates. Molecular analysis of the MBL determinant showed the presence of a $bla_{IMP-13}$ gene carried on a gene cassette inserted in a class 1 integron which also contained an *aacA4* aminoglycoside resistance cassette encoding an AAC(6′)-Ib enzyme. The $bla_{IMP-13}$-containing integron and its genetic environment appeared to be similar to those found in *P. aeruginosa* isolates producing IMP-13 from a hospital in Rome. The $bla_{IMP-13}$ gene was not transferable by conjugation and was apparently carried on the chromosome. The outbreak was coincidental with a shortage of nursing personnel, and resolution was apparently associated with reinstatement of nursing personnel and reinforcement of general infection control practices within the intensive care unit. To our best knowledge this is the first description of a nosocomial outbreak of relatively large size caused by an IMP-producing gram-negative pathogen in Europe.**

Acquired metallo-β-lactamases (MBLs) are emerging resistance determinants in *Pseudomonas aeruginosa* and other gram-negative pathogens (1, 17). These enzymes can hydrolyze most β-lactams, including carbapenems, and can confer a broad-spectrum β-lactam resistance phenotype to the bacterial host, which is not reversible by conventional β-lactamase inhibitors (1, 11). Originally thought to be uncommon and restricted to some geographical areas, acquired MBLs are presently known to be widespread, and at least four different types of these enzymes, IMP, VIM, SPM, and GIM, have been identified (2, 17, 27).

The IMP-type enzymes were detected first, and several different variants of these enzymes are currently known (http://www.lahey.org/studies/other.asp#table 1). They were originally reported in Japan in the early 1990s (18, 33). In that area, IMP-type enzymes apparently represent the most common type of acquired MBL among gram-negative nosocomial pathogens (23), and IMP producers have been involved in nosocomial outbreaks (7, 22). In Europe, although a number of IMP-type variants have been detected since the late 1990s (3, 5, 20, 25, 31), these enzymes appear to be considerably less common than VIM-type enzymes (6, 8, 13, 17, 26, 29), and, to our best knowledge, no important outbreaks of IMP producers have been reported to date.

In this paper we describe a large nosocomial outbreak caused by a multidrug-resistant (MDR) *P. aeruginosa* isolate producing an IMP-type enzyme, IMP-13, which occurred in a large tertiary care and clinical research hospital in southern Italy.

## MATERIALS AND METHODS

**Bacterial isolates.** All *P. aeruginosa* isolates described in this work were from inpatients at a general intensive care unit (ICU) of the "Casa Sollievo della Sofferenza" Istituto di Ricovero e Cura a Carattere Scientifico Hospital, a 1,180-bed tertiary care and clinical research hospital located in San Giovanni Rotondo in southern Italy. Identification of isolates was carried out using the GNI card of the Vitek II System (bioMérieux, Rome, Italy).

**In vitro susceptibility testing.** The in vitro antimicrobial susceptibility of *P. aeruginosa* clinical isolates was routinely determined using the AST21 panel of the Vitek II system (bioMérieux). In isolates subjected to further phenotypic and molecular characterization, MICs were also determined by a microdilution broth method according to the indications of the National Committee for Clinical Laboratory Standard (NCCLS) (15). Results of susceptibility testing were interpreted according to the NCCLS guidelines (16). *P. aeruginosa* ATCC 27853 was always used as a quality control in susceptibility testing.

**Detection of β-lactamase activity.** Phenotypic screening for MBL production was carried out by the Etest MBL assay (AB Biodisk, Solna, Sweden), under the conditions recommended by the test manufacturer, and by the EPI microdilution test as described previously (14). Spectrophotometric assays for detection of MBL activity were carried out as described previously (10), using 150 μM imipenem as substrate and 5 mM EDTA (final concentration) in inhibition assays. Analytical isoelectric focusing (IEF) for detection of β-lactamase activities in bacterial extracts was carried out as described previously, using the nitrocefin chromogenic substrate (10). *P. aeruginosa* ATCC 27853 and *P. aeruginosa* 32SM (an imipenem-resistant clinical isolate from our collection which lacks OprD and constitutively produces the AmpC enzyme but does not produce any MBL enzyme) were used as negative controls for MBL detection. *P. aeruginosa*

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TABLE 1. Phenotypic and genotypic features of the seven MDR *P. aeruginosa* isolates involved in the outbreak and of two carbapenem-resistant sporadic isolates collected from the same ward before the outbreak[e]

| Isolate | Date | Specimen[a] | MIC (μg/ml) | | | | | | | | | | IEF[b] | Etest[c] | EPI[d] | PFGE profile |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | TZP | CAZ | FEP | ATM | IPM | MEM | GEN | TOB | AMK | CIP | | | | |
| MV461 | Feb. '03 | BA | 32 | >64 | >64 | 16 | 32 | 32 | >16 | >16 | 2 | >4 | 8.0 | 32/8 | 32/2 | A |
| AV216 | Feb. '03 | BA | 32 | >64 | >64 | 16 | 32 | 32 | >16 | >16 | 2 | >4 | 8.0 | 32/12 | 32/2 | A |
| AV78 | Mar. '03 | IEP | >128 | >64 | >64 | >32 | 64 | 32 | >16 | >16 | 2 | >4 | 8.0 | 64/32 | 64/4 | A2 |
| AV214 | Mar. '03 | BA | 32 | >64 | >64 | 16 | 32 | 32 | >16 | >16 | 2 | >4 | 8.0 | 32/8 | 32/2 | A |
| MV460 | Mar. '03 | BA | >128 | >64 | >64 | 16 | 32 | 32 | >16 | >16 | 2 | >4 | 6.2–8.0 | 32/16 | 32/8 | A1 |
| AV70 | Mar. '03 | BA | 32 | >64 | >64 | 16 | 32 | 32 | >16 | >16 | 2 | >4 | 8.0 | 32/8 | 32/0.5 | A |
| AV65 | Apr. '03 | CS | 32 | >64 | >64 | 16 | 32 | 32 | >16 | >16 | 2 | >4 | 8.0 | 32/8 | 32/0.5 | A |
| OV160 | Oct. '02 | BA | 32 | >64 | 8 | 16 | 32 | 16 | 2 | ≤1 | 1 | 2 | 8.0 | 32/24 | 32/16 | B |
| OV184 | Oct. '02 | BA | 32 | 2 | 8 | 16 | 16 | 2 | 4 | ≤1 | 1 | ≤0.25 | 8.0 | 32/24 | 16/8 | C |

[a] BA, bronchial aspirate; IEP, intrahepatic drainage; CS, conjunctival swab.
[b] Isoelecric points of β-lactamase bands detected in crude extracts.
[c] Imipenem MICs (in micrograms/milliliter) detected by Etest MBL either in absence or in the presence of EDTA.
[d] Imipenem MICs (in micrograms/milliliter) detected by the EPI microdilution test in absence or in the presence of the chelating mix containing EDTA plus 1,10-phenanthroline.
[e] TZP, piperacillin-tazobactam; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; CIP, ciprofloxacin.

101/1477 (producing IMP-1) (9) and *P. aeruginosa* VR-143/97 (producing VIM-1) (10) were used as positive controls for MBL detection.

**Molecular analysis of MBL genes and of their genetic context.** Multiplex PCR for detection of $bla_{IMP}$ and $bla_{VIM}$ MBL genes was carried out using the IMP-DIA (forward, 5′-GGA ATA GAG TGG CTT AAT TCT C; reverse, 5′-GTG ATG CGT CYC CAA YTT CAC T) and VIM-DIA (forward, 5′-CAG ATT GCC GAT GGT GTT TGG; reverse, 5′-AGG TGG GCC ATT CAG CCA GA) primers, as described previously (12, 14). The same control strains used in phenotypic assays for MBL production (see above) were used for quality control of PCR assays. Genomic DNA was extracted from *P. aeruginosa* as described previously (10). Plasmid DNA extraction was carried out by the alkaline lysis method (21). Southern blot hybridizations were carried out on dried gels, as described previously (30). The probe used for hybridization experiments was a PCR-generated fragment comprising the entire $bla_{IMP-13}$ coding sequence, obtained with primers IMP-13 forward (5′-ATG AAG AAA TTA TTT GTT TTA TG) and IMP-13 reverse (5′-TTA GTT ACT TGG TGA TGA TG) and labeled with $^{32}P$ by the random priming technique using a commercial kit (Rediprime II DNA Labeling System; Amersham Biosciences, Cologno Monzese, Italy). Characterization of the variable region of class 1 integrons and of integron 5′-flanking sequences was carried out by a PCR mapping and sequencing approach, as described previously (20, 26). The sequences of PCR products were determined on both strands directly on PCR products.

**Genotyping by pulsed-field gel electrophoresis (PFGE).** Macrorestriction profiles of genomic DNA were analyzed by means of the Gene Path Procedure (Bio-Rad Laboratories, Richmond, Calif.) using the #3 pathogen group reagent kit and the restriction enzyme SpeI. DNA fragments were analyzed by electrophoresis using 1% agarose gels in 0.5× Tris-borate-EDTA buffer with the Gene Path system (Bio-Rad) using the following parameters: temperature, 14°C; field strength, 6 V/cm for 20 h; angle, 120°; pulse times, 5.3 to 34.9 s. Bacteriophage λ concatemers (Bio-Rad) were used as DNA size markers. After electrophoresis the restriction fragments were stained with ethidium bromide and visualized under UV light. Clonal relatedness based on the PFGE patterns was interpreted according to the criteria proposed by Tenover et al. (24).

**Gene transfer experiments.** Conjugation experiments were performed on Mueller Hinton (MH) agar plates (Difco Laboratories, Detroit, Michigan). *Escherichia coli* MKD-135 (*argH rpoB18 rpoB19 recA rpsL*) or *P. aeruginosa* 10145/3 (an *rpoB his* derivative of strain ATCC10145$^T$) were used as recipients in conjugation experiments. The initial donor/recipient ratio was 0.1. Mating plates were incubated at 37°C for 7 h. *E. coli* transconjugants were selected on MH agar containing 4 μg/ml ceftazidime and 300 μg/ml rifampin. *P. aeruginosa* transconjugants were selected on MH agar containing 4 μg/ml imipenem and 300 μg/ml rifampin. With either recipient, the detection sensitivity of the assay was $\geq 5 \times 10^{-8}$ transconjugants/recipient.

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper have been submitted to the EMBL/GenBank sequence database and assigned accession number AJ628135.

## RESULTS AND DISCUSSION

**Description of the outbreak.** In February 2003 a *P. aeruginosa* isolate showing an unusual MDR phenotype (isolate MV461) was isolated from the lower respiratory tract of a mechanically ventilated patient from one of the general ICUs of a large tertiary care and clinical research hospital located in San Giovanni Rotondo (southern Italy). MV461 was resistant to carbapenems (imipenem and meropenem), extended-spectrum cephalosporins (ceftazidime and cefepime), and gentamicin, tobramycin, and fluoroquinolones (ciprofloxacin and levofloxacin), while it retained susceptibility to piperacillin-tazobactam and amikacin. Before this episode, carbapenem-resistant *P. aeruginosa* isolates had only been reported sporadically at the clinical microbiology laboratory of that hospital, and they had never shown such a complex MDR phenotype. At the time of isolation of MV461, the patient was receiving broad-spectrum antibiotic coverage with meropenem plus teicoplanin.

*P. aeruginosa* isolates showing the same MDR phenotype as MV461 were repeatedly isolated from the lower respiratory tract of that patient and, during the following 5-month period, a total of 86 *P. aeruginosa* isolates with an identical or very similar MDR phenotype (the only difference being represented by additional resistance to piperacillin-tazobactam in some cases) were isolated from 14 additional inpatients from that ICU. All those patients yielded the MDR isolates from lower respiratory tract specimens, and 9 of them also yielded isolates from other specimens, including urine ($n = 7$), blood ($n = 2$), or other specimens ($n = 2$).

During the outbreak period (February to June 2003) imipenem-susceptible or -intermediate *P. aeruginosa* isolates were also isolated from inpatients from that ICU (a total of 31 isolates from eight patients, all different from those yielding the MV461-like MDR isolates). Of these isolates, some were uniformly susceptible to anti-pseudomonal agents while others exhibited variable resistance patterns, including ciprofloxacin, gentamicin, tobramycin, and β-lactams, although they never showed an MDR phenotype comparable to that of MV461.

The outbreak subsided in July 2003. Thereafter, MV461-like MDR *P. aeruginosa* isolates were no longer recovered from that ICU or from other hospital wards, except for occasional isolates from the respiratory tract of the index patient who has been kept in the ICU on mechanical ventilation since then (the patient was comatose due to a stroke). The outbreak was coincidental with a shortage of nursing personnel, and resolution was apparently associated with reinstatement of nursing personnel and reinforcement of general infection control practices within the ICU. No other epidemiological, environmental, or behavioral factors associated to the outbreak could be identified.

**Phenotypic and genotypic characterization of the MDR *P. aeruginosa* isolates.** Of the MDR *P. aeruginosa* isolates involved in the outbreak, only seven nonreplicate isolates collected during the first 2 months of the outbreak (including the index isolate MV461) were preserved by the clinical microbiology laboratory of the hospital and could be subjected to further characterization. Two imipenem-resistant *P. aeruginosa* isolates isolated from respiratory specimens of inpatients from the same ICU before the outbreak (in late 2002), which did not show a MDR phenotype similar to that of MV461, were also available and were investigated for comparison.

The MDR isolates involved in the outbreak exhibited imipenem MICs of 32 to 64 μg/ml and meropenem MICs of 32 μg/ml (Table 1). The Etest MBL, performed according to the manufacturer's recommendations, showed a reduction of the imipenem MICs in the presence of EDTA with all the MDR isolates (Table 1). However, the extent of MIC reduction (two- to fourfold) (Table 1) was not to be considered significant for MBL production according to the interpretive criteria recommended by the manufacturer. On the other hand, the EPI microdilution test exhibited an ≥8-fold reduction of imipenem MICs in the presence of the chelating mix with each of these isolates, suggesting production of MBL activity (14) (Table 1). Enzyme assays revealed the presence of EDTA-inhibitable carbapenemase activity in crude extracts of the seven MDR isolates involved in the outbreak (>140 nmol/min · mg of protein). Analytical IEF revealed the presence of β-lactamase bands of alkaline pI (>8) in all isolates and the presence of an additional β-lactamase band of acidic pI in one of them (Table 1).

The macrorestriction profiles of the SpeI-digested genomic DNAs of the seven MDR *P. aeruginosa* isolates involved in the outbreak were either identical to each other (five isolates, including the index isolate MV461) or different by no more than four bands (Fig. 1), revealing clonal relatedness. Interestingly, the five isolates showing an identical PFGE profile (profile A) also exhibited identical resistance patterns, while the two isolates showing some differences from the dominant profile (profiles A1 and A2) exhibited minor differences in the resistance patterns and, for one of them, also in the β-lactamase profile detected by IEF (Table 1). Although genotypic analysis could not be performed on all the nonreplicate isolates involved in the outbreak (since only those from the first period had been preserved), it is highly likely that all the MDR isolates involved in the outbreak were clonally related, given the consistency of their resistance phenotype and their overall epidemiological relationships. The PFGE profiles of the two imipenem-resistant sporadic isolates were notably different (by more than seven bands) from each other and from those of the MDR isolates involved in the outbreak (data not shown), suggesting that these isolates were not related to the strain causing the outbreak.

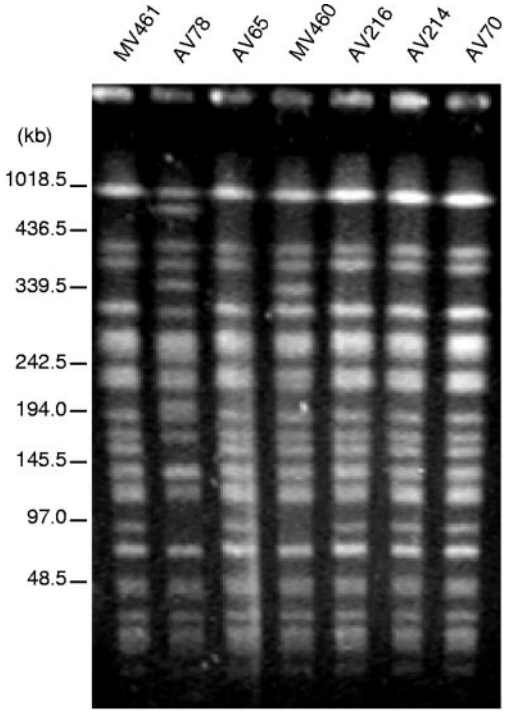


FIG. 1. SpeI PFGE profiles of the seven MDR *P. aeruginosa* isolates involved in the outbreak that occurred in the ICU and of the two sporadic imipenem-resistant *P. aeruginosa* isolates isolated from the same ward in the period preceding the outbreak. The names of isolates are the same as in Table 1. DNA size standards are indicated in kilobases on the left.

**Characterization of the MBL determinants and of their genetic support.** Multiplex PCR analysis of the seven MBL-producing *P. aeruginosa* isolates involved in the outbreak, using primers suitable for detection of $bla_{IMP}$ and $bla_{VIM}$ genes, yielded, from each of them, a product whose size (0.36 kb) was consistent with the presence of a $bla_{IMP}$ MBL determinant.

PCR amplification of the variable region of class 1 integrons, using primers INT/5CS and INT/3CS designed on the integron 5′- and 3′-conserved segments (CS) (20), yielded a 2.2-kb amplification product from each of the seven MBL producers. Sequencing of the amplification product obtained from MV461 revealed the presence of a $bla_{IMP-13}$ cassette followed by an *aacA4* cassette inserted between the 5′- and 3′-CS of a class 1 integron (Fig. 2). Restriction profiling of the amplification products obtained from the other six isolates, using ClaI, AluI, RsaI, and HindIII, yielded identical profiles with all the isolates (data not shown), which were fully consistent with the sequence of the amplification product obtained from MV461, suggesting that the variable region of the $bla_{IMP-13}$-containing integrons were identical in all seven MDR isolates. Sequencing the genomic region located upstream of the $bla_{IMP-13}$-containing integron of MV461 revealed a 5′-CS typical of class 1 integrons inserted into the *res* site of a Tn*5051*-like transposon (Fig. 2). The $bla_{IMP-13}$ cassette, the cassette array, and the

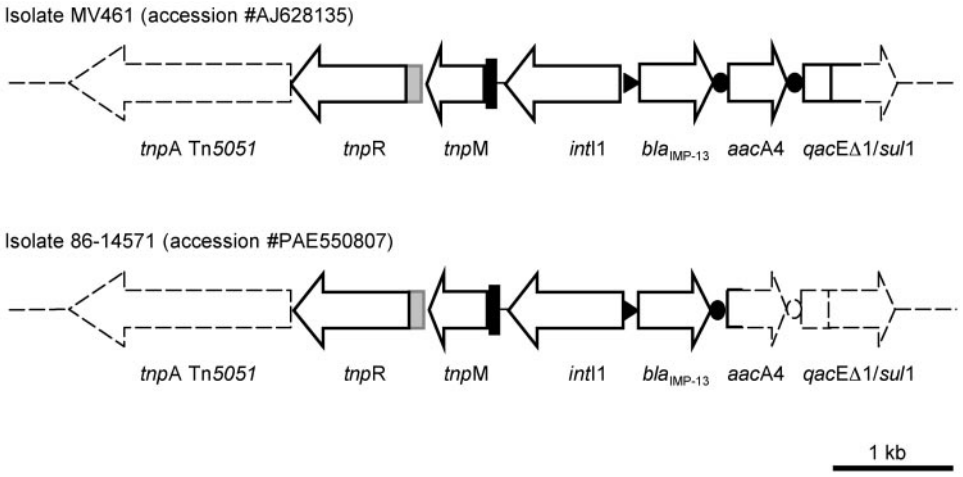


FIG. 2. Schematic representation of the structure of the class 1 integron carrying the $bla_{IMP-13}$ cassette from isolate MV461 and of its genetic context. Open reading frames are indicated by arrows; the *attI* sites are indicated by triangles; the *attC* sites (59-bp elements) are indicated by filled circles. The 25-bp inverted repeat sequence ($IR_i$) and the *res* site are shown as black and gray rectangles, respectively. The structure of the $bla_{IMP-13}$–containing integron from IMP-13-producing *P. aeruginosa* isolates isolated in Rome within the SENTRY surveillance program (86-14571) (25, 26) is also shown for comparison. Filled lines indicate the sequenced regions, as reported in database entries.

region upstream the $bla_{IMP-13}$-containing integron were identical to those found in IMP-13-producing *P. aeruginosa* isolates isolated in Rome within the SENTRY surveillance program (25, 26) (Fig. 2).

Conjugation experiments, carried out using either an *E. coli* or a *P. aeruginosa* recipient, consistently failed to demonstrate horizontal transfer of the MBL gene from MV461 and also from MV460 and AV78 (i.e., the two isolates that showed a PFGE profile not identical to MV461). Plasmid DNA preparations from the seven MDR isolates revealed large plasmid forms (apparently identical) in six of them. However, in Southern blot experiments carried out with plasmid DNA preparations and with whole genomic DNA preparations extracted by standard methods, using a $bla_{IMP-13}$ probe a positive hybridization signal was only observed with the band of chromosomal DNA (Fig. 3 and data not shown), suggesting a chromosomal location of the $bla_{IMP-13}$-containing integron.

**Concluding remarks.** IMP-type MBLs appear to be overall less common than VIM-type enzymes in Europe (3, 31), and Italy is the only European country where *P. aeruginosa* isolates producing IMP-type enzymes have been thus far reported (4, 26). Unlike VIM-producing *P. aeruginosa*, for which large nosocomial outbreaks have been reported in Greece and Italy (8, 19, 28), to our best knowledge this is the first report of a relatively large nosocomial outbreak caused by *P. aeruginosa* producing an IMP-type enzyme. Present findings, therefore, indicate that nosocomial outbreaks of *P. aeruginosa* producing IMP-type enzymes could become an emerging reality in Europe.

In this case a single clone was apparently responsible for the outbreak, which occurred within an ICU setting. In that clone, the $bla_{IMP-13}$ gene was apparently carried on the chromosome and was not transferable by conjugation, as it is often observed for acquired MBL determinants in *P. aeruginosa* (17). Interestingly, the $bla_{IMP-13}$ gene cassette was inserted into an integron whose cassette array and genetic context were apparently identical to those found in IMP-13-producing *P. aeruginosa* isolated in Rome (25), suggesting that either the same clone or the same element are spreading in different Italian hospitals. It would be interesting to compare those strains to ascertain this point.

There was no obvious factor to which the outbreak could be directly related, except for a coincidental shortage of nursing personnel. The outbreak resolution was apparently associated

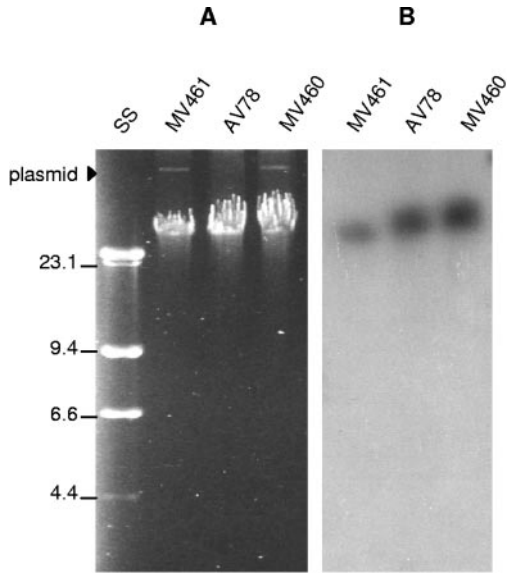


FIG. 3. (A) Agarose gel electrophoresis of total genomic DNA extracted from isolates MV461 (index strain, PFGE profile A), AV78 (PFGE profile A2), and MV460 (PFGE profile A1). Plasmid DNA bands, visible in the DNA preparations from MV461 and MV460, are indicated. DNA size standards (SS) are indicated in kilobases on the left. (B) Results of Southern blot analysis of the gel shown in panel A after hybridization with a $bla_{IMP-13}$ probe. Results obtained with the four other isolates showing PFGE profile A were identical to those observed with MV461 and are not shown.

to reinstatement of nursing personnel associated to a reinforcement of general measures for prevention of nosocomial spread of resistant pathogens.

Concerning detection of MBL production, the IMP-13-producing strain responsible for this outbreak exhibited a reduction of imipenem MICs with both the EPI test and the Etest MBL assay. However, the reduction of imipenem MICs (two- to fourfold) observed with Etest was not suggestive for MBL production according to the recommendations of the test manufacturer (32). A similar behavior might be related to this particular strain, although it should be noted that the imipenem MIC reduction previously reported for IMP-13-producing *P. aeruginosa* did not exceed the interpretative breakpoint of eightfold (25). This finding underscores the notion that phenotypic tests for MBL detection could exhibit variable sensitivity depending on the type of test, the nature of the enzyme, and the nature of the bacterial host, which could be a relevant issue in surveillance programs.

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