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Outbreak of Extended-Spectrum β-Lactamase VEB-1-Producing Isolates of *Acinetobacter baumannii* in a French Hospital

Laurent Poirel,[1] Olivier Menuteau,[1] Nathalie Agoli,[2] Christian Cattoen,[2] and Patrice Nordmann[1]*

Service de Bactériologie-Virologie, Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine Paris-Sud, 94275 Le Kremlin-Bicêtre,[1] and Service de Biologie Clinique, CHG Valenciennes,[2] France

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Twelve clonally related and multidrug-resistant *Acinetobacter baumannii* isolates were recovered during a 4-month period from 12 patients hospitalized at the Valenciennes Hospital in France. Antibiograms determined by the double-disk diffusion technique on cloxacillin-containing plates detected a clavulanic acid-inhibited extended-spectrum β-lactamase (ESBL). PCR and sequencing identified the gene encoding the Ambler class A ESBL VEB-1. This gene was located on the chromosome and was part of a class 1 integron identical to that previously identified in *Pseudomonas aeruginosa* isolates from Thailand. Additionally, seven clonally related bla_{VEB-1}-positive *A. baumannii* strains were identified in the immediate environment of the hospitalized patients. This is the first report of the ESBL VEB-1 in *Acinetobacter* spp. and the first description of VEB-1-producing strains as a source of an outbreak occurring outside Southeast Asia. This report underlines the difficulty of the identification of ESBLs in *A. baumannii*.

Acinetobacter baumannii is an opportunistic pathogen involved in outbreaks occurring in intensive care units (ICUs) (2, 14). It is an important source of nosocomial septicemia, pneumonia, and urinary tract infections (4). Reports of multidrug-resistant isolates have increased during the last decade, probably as a result of the extensive use of broad-spectrum antibiotics (1). In many cases, these multidrug-resistant isolates are resistant to expanded-spectrum cephalosporins and carbapenems (1, 2, 28). Recent studies report that carbapenem-hydrolyzing β-lactamases of Ambler class B (metalloenzymes) and Ambler class D (oxacillinases) are sources of multidrug resistance in *A. baumannii* (3, 5, 24, 27, 30). However, most of the expanded-spectrum β-lactamases of gram-negative organisms are the clavulanic acid-inhibited extended-spectrum β-lactamases (ESBLs) of Ambler class A that have been reported extensively in members of the family *Enterobacteriaceae* (19). They usually confer resistance to cefotaxime, ceftriaxone, ceftazidime, and the monobactam aztreonam but do not confer resistance to imipenem (19).

Most of these ESBLs that are disseminated worldwide are structurally related to the narrow-spectrum TEM- and SHV-type β-lactamases, but none of them has been detected so far in *A. baumannii* (19).The non-TEM, non-SHV derivative ESBL PER-1 is the only known ESBL in *A. baumannii* and has been detected in Turkish and French isolates (21, 31, 32). An epidemiological survey performed in Turkey in 1996 identified the spread of PER-1-positive *A. baumannii* isolates (32), and infections with these PER-1-positive isolates have been associated with a higher risk of mortality (31).

Another non-TEM, non-SHV ESBL, VEB-1, has been detected in *Enterobacteriaceae* and *Pseudomonas aeruginosa* strains from Southeast Asia (7, 8, 15–17, 23). Unlike most of the ESBL genes, bla_{VEB-1} is part of a gene cassette and is located in class 1 integrons of various structures (7, 8, 15–17, 23). Integrons are genetic structures responsible for the expression of cassette-associated and mobile resistance genes (25).

The aim of the present study was to analyze the molecular mechanisms involved in the β-lactam resistance of multidrug-resistant and nosocomial *A. baumannii* isolates. This study identified for the first time the ESBL VEB-1 in *A. baumannii*. It shows also that (i) ESBL-positive *A. baumannii* isolates may be responsible for nosocomial outbreaks, (ii) ESBLs may contribute to the multidrug resistance of *A. baumannii* isolates, and (iii) that ESBL production may remain undetected in this species.

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MATERIALS AND METHODS

Bacterial isolates. *A. baumannii* clinical and environmental isolates (strains 1 to 20) were identified by using the API 32GN system (bioMérieux SA, Marcy l'Etoile, France). Electrocompetent *Escherichia coli* DH10B (GIBCO BRL, Life Technologies, Cergy Pontoise, France) was used as the recipient strain in transformation experiments. Rifampin-resistant *E. coli* HB101 was used as the host in conjugation experiments (23). *E. coli* MG-1, which carries the bla_{VEB-1} gene, was used as a VEB-1-producing reference strain (23). *E. coli* K-12 DNA, which generates seven I-*Ceu*I restricted fragments of 2,460, 700, 670, 530, 130, 92, and 44 kb, was used as a size marker in pulsed-field gel electrophoresis (PFGE) experiments. Reference strain *A. baumannii* CIP7034^T (Institut Pasteur Strain Collection, Paris, France) was used as a nonclonally related strain in the PFGE experiments, and an in vitro-obtained rifampin-resistant derivative was used in conjugation experiments.

Susceptibility testing and screening for ESBL-producing strains. The antibiotic susceptibilities of the *A. baumannii* isolates were first determined by the disk diffusion method on Mueller-Hinton (MH) agar plates with antibiotic-containing disks (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France), and the results

* Corresponding author. Mailing address: Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin-Bicêtre cedex, France. Phone: 33-1-45-21-36-32. Fax: 33-1-45-21-63-40. E-mail: nordmann.patrice@bct.ap-hop-paris.fr.

were interpreted according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (www.sfm.fr). The double-disk synergy test was performed with cefepime, ceftazidime, and ticarcillin-clavulanic acid disks on MH agar plates; and the results were interpreted as described previously (10). To counteract the effect of high-level expression of the naturally produced AmpC-type β-lactamase of *A. baumannii*, double-disk synergy tests were also performed on cloxacillin (200 μg/ml)-containing plates (6).

The MICs of the β-lactams were determined by an agar dilution technique on MH agar plates with or without cloxacillin (200 μg/ml) with an inoculum of 10^4 CFU per spot, as described previously (20). MIC results were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (18).

IEF analysis. Cultures of *A. baumannii* isolates were grown overnight at 37°C in 10 ml of Trypticase soy (TS) broth. Analytical isoelectric focusing (IEF) was performed with an ampholine polyacrylamide gel, as described previously (20).

PCR-based amplification of β-lactamase genes and class 1 integrons and sequencing. Under standard PCR conditions (26), a series of primers was used for detection of Ambler class A β-lactamase genes. Detection of genes coding for β-lactamases TEM, SHV, PER-1 or PER-2, VEB-1, and GES-1 was performed as described previously (7, 8). Detection of an *ampC*-type gene of *A. baumannii* was performed with primers preAB-1 (5′-ACAGAGGAGCTAATCATGCG-3′) and preAB-2 (5′-GTTCTTTTAAACCATATACC-3′), which hybridized to the internal part of this β-lactamase gene. For each reaction, 0.5 μg of whole-cell DNA of the *A. baumannii* isolates was used. The primers used for detection of class 1 integrons (primers 5′-CS and 3′-CS [12]) hybridized to sequences located in the 5′ and 3′ conserved segments. Combinations of primer 5′-CS or 3′-CS and bla_{VEB-1}-specific primers (primers 5′-CS and VEB-B or primers 3′-CS and VEB-F [15, 17]) were also used for determination of the genetic contents of the class 1 integrons. Additionally, since the *oxa-10* and *arr-2* genes had been associated with the bla_{VEB-1} gene in *E. coli* MG-1 and *P. aeruginosa* isolates (7, 16), their positions relative to that of the bla_{VEB-1} gene were determined by PCR with primers specific for bla_{VEB-1} (primers VEB-1A and VEB-1B and primers VEB-INV4F and VEB-INFV3B), for bla_{OXA-10} (primers OXA-10promB, OPR-1, and OPR-2), and for *arr-2* (primers ARR-2-F and ARR-2-B) (7, 8). Finally, since a bla_{VEB-1}-containing integron had been reported to be bracketed by two IS*26* insertion sequences in *E. coli* MG-1 (15), PCRs with bla_{VEB-1}-specific primers and IS*26*-specific primers were also performed (16). Sequencing reactions were performed with the same bla_{VEB-1}-specific primers and an automated sequencer (ABI 377; Applied Biosystems, Foster City, Calif.). The nucleotide and deduced amino acid sequences were analyzed with software available over the Internet (22, 23).

PFGE. PFGE analysis was done according to the instructions of the manufacturer (Bio-Rad). In brief, whole-cell DNA of the *A. baumannii* isolates was digested with the *Apa*I restriction enzyme overnight at 25°C (9). Electrophoresis was performed with a CHEF DRII apparatus (Bio-Rad) through a 1% agarose gel in 0.5× Tris-borate-EDTA buffer. Migration conditions were as follows: temperature, 14°C; voltage, 5 V/cm; and switch angle, 120°, with one linear switch ramp of 5 to 20 s for 24 h. The ethidium bromide-stained gel was photographed under UV illumination. A bacteriophage λ DNA ladder (Bio-Rad) was used as a DNA molecular weight marker. The chromosomal fingerprints were compared by eye and assigned to PFGE types and subtypes (29).

Hybridizations. The I-*Ceu*I restriction enzyme (Ozyme; New England Biolabs), which digests a 26-bp sequence in the *rrn* genes for the 23S large-subunit rRNA, was used to search the chromosome to determine whether the β-lactamase gene has a chromosomal location (13); and the fragments were separated by PFGE. The sizes of the I-*Ceu*I-generated fragments of *A. baumannii* clinical isolates AYE and 13 and of reference strain CIP7034^T were determined by comparison with those of *E. coli* K-12. After Southern transfer onto a nylon membrane (Hybon N^+; Amersham Pharmacia Biotech, Orsay, France) (26), the DNAs were UV cross-linked (Stratalinker; Stratagene) and hybridized successively with two probes: a 1,504-bp PCR-generated probe specific for the 16S and 23S rRNA genes (8) and a 650-bp probe specific for bla_{VEB-1} (23). Labeling and signal detection were carried out according to the instructions of the manufacturer (Amersham Pharmacia Biotech).

Conjugation, electroporation, and plasmid DNA analysis. Conjugation experiments were performed between *A. baumannii* clinical isolate AYE and *E. coli* HB101, which were resistant to rifampin, and in vitro-obtained rifampin-resistant *A. baumannii* CIP7034^T in solid and liquid media at 37°C, as reported previously (20). Transconjugants were selected on TS agar plates containing 300 μg of rifampin per ml and 150 μg of ticarcillin per ml. The plasmid DNA of the *A. baumannii* isolates was extracted as described previously (7, 22). Plasmid extracts were electroporated into *E. coli* DH10B, and recombinant strains were selected on ceftazidime (2 μg/ml)-containing TS agar plates.

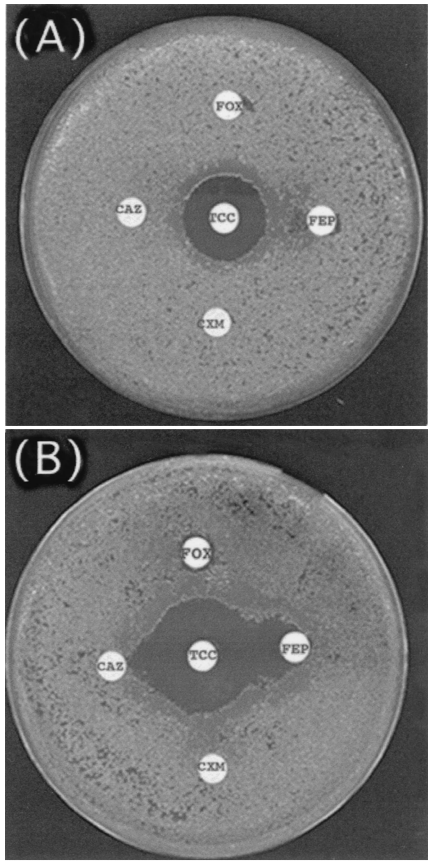

FIG. 1. Double-disk synergy test with bla_{VEB-1}-positive *A. baumannii* strain AYE. MH agar plates contained either no cloxacillin (A) or 200 μg of cloxacillin per ml (B). The disks tested contained ceftazidime (CAZ), cefuroxime (CXM), ticarcillin-clavulanic acid (TCC), cefoxitin (FOX), and cefepime (FEP).

RESULTS

Preliminary PCR detection of β-lactamase gene. A multidrug-resistant *A. baumannii* isolate, isolate AYE, was recovered from a patient with a urinary tract infection. The patient was a 61-year-old man who was hospitalized at the Bicêtre Hospital (Le Kremlin-Bicêtre, France) in October 2001. He had been transferred directly from the ICU of the Valenciennes Hospital (a 1,000-bed facility in the north part of France), where he had been hospitalized for pneumonia and where an antibiotic regimen containing imipenem and gentamicin had been given. Preliminary antibiotic susceptibility testing by disk diffusion showed that *A. baumannii* isolate AYE was resistant to all β-lactams except imipenem, piperacillin-tazobactam, and ticarcillin-clavulanate. It was resistant to other antibiotics such as fluoroquinolones, aminoglycosides, chloramphenicol, and tetracycline and was of intermediate susceptibility to rifampin (data not shown). A synergy between ceftazidime- or cefepime- and clavulanate-containing disks was evidenced only when cloxacillin-containing agar plates were used (Fig. 1). PCR experiments with primers specific for bla_{TEM}, bla_{SHV}, bla_{VEB-1}, bla_{GES-1}, bla_{PER-1}, and $bla_{CTX-M-1}$ were performed and gave a positive result for a bla_{VEB-1}-like gene. Sequence analysis of this bla_{VEB-1}-like gene revealed a 100%

TABLE 1. MICs of β-lactams for *A. baumannii* AYE determined on MH agar and cloxacillin-containing MH agar plates

β-Lactam(s)[a]	MIC (μg/ml) on:	
	MH agar plates	Cloxacillin-containing MH agar plates
Amoxicillin	>512	>512
Amoxicillin + CLA	>512	8
Ticarcillin	>512	>512
Ticarcillin + CLA	32	16
Piperacillin	256	64
Piperacillin + TZB	8	0.06
Cephalothin	>512	>512
Cefuroxime	>512	256
Cefotaxime	512	256
Cefotaxime + CLA	64	16
Ceftazidime	>512	>512
Ceftazidime + CLA	64	16
Cefoxitin	>512	64
Cefepime	512	64
Cefepime + CLA	256	4
Cefpirome	512	256
Cefpirome + CLA	256	16
Moxalactam	64	64
Aztreonam	>512	>512
Imipenem	1	0.5

[a] CLA, clavulanic acid at a fixed concentration of 2 μg/ml; TZB, tazobactam at a fixed concentration of 4 μg/ml.

identity of its DNA with that of bla_{VEB-1}. In addition and as expected, PCR with primers located at the ends of the *ampC*-type β-lactamase gene of *A. baumannii* gave a 1,242-bp DNA fragment, indicating the presence of this naturally occurring β-lactamase gene in *A. baumannii* AYE.

MICs of β-lactams and IEF analysis. The MICs of ceftazidime and cefotaxime for *A. baumannii* AYE were lowered when clavulanic acid was added, which is consistent with expression of the ESBL VEB-1 (Table 1). The contribution of VEB-1 to resistance to expanded-spectrum cephalosporins was demonstrated by using cloxacillin-containing MH agar plates (Table 1).

IEF analysis showed that *A. baumannii* AYE expressed several β-lactamases with pI values of 6.3, 7.4, and 8.5. The pI value of 6.3 likely corresponded to that of OXA-10 (7, 8), and the pI value of 7.4 corresponded to that of VEB-1, whereas the pI value of 8.5 corresponded to that of an AmpC-type cephalosporinase (3).

Identification of bla_{VEB-1}-positive integron. Fragments were obtained by PCR with primers 5′-CS and 3′-CS for detection of class 1 integrons and bla_{VEB-1}-specific primers, indicating that bla_{VEB-1} is part of a class 1 integron. Taking into account the sizes of the amplified fragments, the structure of the integron of *A. baumannii* AYE was identical to that found in most of the *P. aeruginosa* isolates from Thailand, which also contained bla_{OXA-10} and rifampin resistance-encoding *arr-2* genes (Fig. 2) (7). As in most of the Thai *P. aeruginosa* isolates (7), insertion sequence IS*1999* was also located inside the class 1 integron structure, interrupting the integron recombination site *attI*. A PCR experiment also identified an IS*26*-like element upstream of the bla_{VEB-1}-positive integron in *A. baumannii* AYE.

Genetic location of bla_{VEB-1} gene. Transfer of the ceftazidime resistance marker by conjugation failed with the *E. coli* and *A. baumannii* recipient strains. Although no plasmid was detected in *A. baumannii* AYE, a suspension containing putative plasmid DNA of *A. baumannii* AYE was used to electroporate *E. coli* DH10B, but no ampicillin-resistant transformant was obtained. The location of bla_{VEB-1} was then determined by using the technique with the endonuclease I-*Ceu*I. Five DNA fragments (2,200, 500, 150, 60, and 40 kb) were generated from the *A. baumannii* CIP7034^T reference strain, whereas six fragments (the five fragments mentioned above plus a 600-kb fragment) were obtained from *A. baumannii* AYE (Fig. 3A). The probe for the rRNA genes hybridized to all DNA fragments except the 2,200-kb fragment for both *A. baumannii* strains (Fig. 3B). Hybridization of restricted DNA of *A. baumannii* AYE with the bla_{VEB-1}-specific probe gave a single signal corresponding to the additional 600-kb fragment, indicating a chromosomal location of bla_{VEB-1} (Fig. 3C).

Retrospective epidemiological survey. No other multidrug-resistant *A. baumannii* isolate was recovered in the ICU of Bicêtre Hospital when *A. baumannii* AYE was isolated. Thus, a retrospective survey was performed at the ICU of the hospital where the patient came from. Twelve additional multidrug-resistant *A. baumannii* isolates from 12 patients were recovered during a 4-month period that surrounded the date of isolation of *A. baumannii* AYE (isolates 1, 2, and 4 to 12 [Table 2] and isolate 13). Most of them suffered from pneumonia. In addition, systematic screening performed during this period gave seven additional multidrug-resistant *A. baumannii* isolates (isolates 14 to 20). These isolates were recovered from ventilators in several ICU rooms (data not shown). PCR with bla_{VEB-1}-specific primers and sequencing of the PCR products revealed that all but one isolate (*A. baumannii* strain 13) possessed a bla_{VEB-1} gene (data not shown). A similar bla_{VEB-1}-positive class 1 integron was identified by PCR analysis with whole-cell DNA of each bla_{VEB-1}-positive strain as the template. The bla_{VEB-1}-positive isolates had the same multiple antibiotic resistance patterns as strain AYE (data not shown).

The PFGE profiles of *Apa*I-restricted DNA of all *A. baumannii* isolates except isolate 13 and the reference strain were identical (Fig. 4). An additional 220-kb fragment was identified in five isolates (strains 5, 6, 11, 15, and 20). Thus, all bla_{VEB-1}-

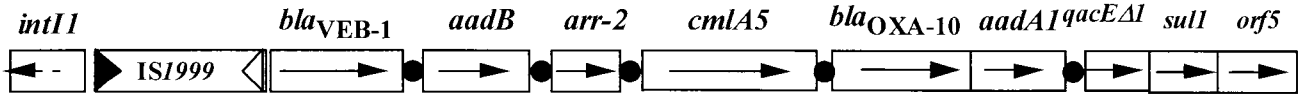

FIG. 2. Schematic representation of the *veb-1* gene cassette-containing integron found in *A. baumannii* AYE. Gene cassettes are shown as boxes, with arrows indicating the orientation of transcription and black circles indicating the 59-base element. The 5′ conserved segment contains the *intI1* gene, which encodes integrase; and the 3′ conserved segment, downstream of the gene cassettes, includes the *qacEΔ1* gene, an antiseptic resistance determinant. The IS*1999* inverted repeats are indicated by filled and empty triangles.

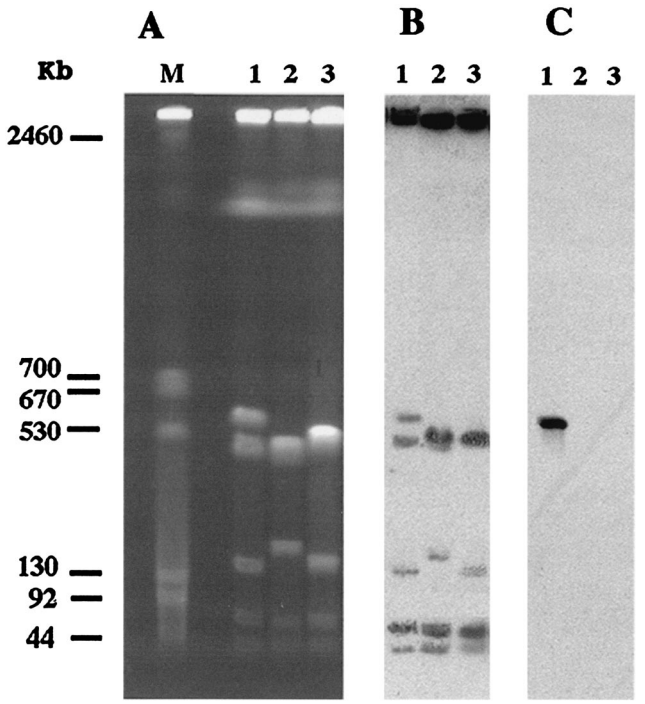

FIG. 3. (A) PFGE profiles of I-*Ceu*I-digested whole-cell DNA of three *A. baumannii* strains. Lanes 1, *A. baumannii* AYE (bla_{VEB-1} positive); lanes 2, *A. baumannii* isolate 13 (bla_{VEB-1} negative); lanes 3, *A. baumannii* reference strain CIP7034^T (bla_{VEB-1} negative); lane M, bacteriophage lambda DNA ladder. Southern hybridization was done with a 16S-23S rRNA gene-specific probe (B) and a bla_{VEB-1}-specific internal probe (C).

positive isolates seemed to be clonally related. They had the same antibiotic resistance pattern, according to disk diffusion susceptibility testing (data not shown).

DISCUSSION

This work describes multidrug-resistant *A. baumannii* isolates as the source of an outbreak in an ICU. Production of an ESBL was demonstrated, whereas dissemination of resistant isolates had already occurred. A 2-week closure of the ICU was the only way to control the outbreak, as demonstrated previously (11).

The clonally related *A. baumannii* isolates produced an unusual Ambler class A ESBL, VEB-1, whereas this enzyme has been identified extensively from members of the family *Enterobacteriaceae* and *P. aeruginosa* isolates in Southeast Asia (7, 8, 15–17, 23). Thus, this work identified this ESBL in *A. baumannii* isolates for the first time (and is the second ESBL found in *A. baumannii*) and represents the first report of an outbreak due to VEB-1-positive gram-negative organisms outside Southeast Asia.

Interestingly, this ESBL gene was identified in a class 1 integron of *A. baumannii* that was encoded by the chromosome, as reported for most of the bla_{VEB-1}-positive *P. aeruginosa* isolates (7). The structure of this bla_{VEB-1}-containing integron was identical to that found in most of the *P. aeruginosa* isolates in a study performed in Thailand (7). The locations of other antibiotic resistance genes in the same integron may contribute to the multidrug resistance of *A. baumannii*. Interestingly, this bla_{VEB-1}-containing integron was bracketed, at least upstream, by insertion sequence IS*26*, as was found for the bla_{VEB-1}-positive integron in *E. coli* MG-1 (16). Thus, the chromosomal location of bla_{VEB-1} in *A. baumannii* may result from a transposition event.

Analysis of this outbreak due to ESBL-producing *A. baumannii* strains raises the threat of endemicity of multidrug-resistant *A. baumannii* isolates, as in Turkey (32). Indeed, a nation-based study performed in that country found that 46% of *A. baumannii* strains produce another ESBL, PER-1 (32).

The spread of ESBL-producing *A. baumannii* isolates may be enhanced by underdetection and underreporting. We show here that the use of cloxacillin-containing plates that inhibit cephalosporinase activity may enhance the ability to detect these organisms in a routine laboratory. Those plates should be used in investigations of outbreaks due to multidrug-resistant *A. baumannii* isolates. Such investigations should be performed, since outbreaks due to *A. baumannii* are usually more difficult to control than those due to members of the family *Enterobacteriaceae* (2, 4). Indeed, the reservoirs of *A. bauman-*

TABLE 2. Clinical features of the bla_{VEB-1}-positive *A. baumannii* isolates

Isolate	Date of isolation (mo-day-yr)	Dates of hospitalization (mo-day-yr)	Source	Underlying disease	Treatment
1	07-01-01	06-08-01 to 07-04-01	Blood	Sepsis	Cefotaxime, ciprofloxacin
2	08-24-01	08-07-01 to 09-12-01	Blood	Pneumonia	Ceftriaxone, ofloxacin
3 (AYE)	10-02-01	09-14-01 to 09-25-01	Urine	Pneumonia	Imipenem, gentamicin
4	10-10-01	09-14-01 to 10-14-01	Blood	Intestinal obstruction	Amoxicillin-CLA,[a] ofloxacin
5	10-10-01	09-28-01 to 10-15-01	Tracheal aspirate	Pneumonia	Imipenem, teicoplanin
6	10-11-01	10-01-01 to 10-18-01	Tracheal aspiration	Sepsis	Piperacillin-tazobactam
7	10-15-01	09-24-01 to 11-02-01	Urine	Pneumonia	Amoxillin-CLA, ofloxacin
8	10-15-01	10-06-01 to 10-15-01	Catheter	Pneumonia	Amoxicillin-CLA, ofloxacin
9	10-15-01	08-01-01 to 03-01-02	Tracheal aspirate	Guillain-Barrè syndrome	Ceftazidime, amikacin
10	10-15-01	10-02-01 to 10-22-01	Urine	Pneumonia	Amoxicillin-CLA, ofloxacin
11	10-22-01	09-16-01 to 10-28-01	Peritonitis fluid	Pneumonia	Imipenem
12	10-26-01	10-04-01 to 10-31-01	Rectal swab	Pneumonia	Teicoplanin, amikacin

[a] CLA, clavulanic acid at a fixed concentration of 2 μg/ml.

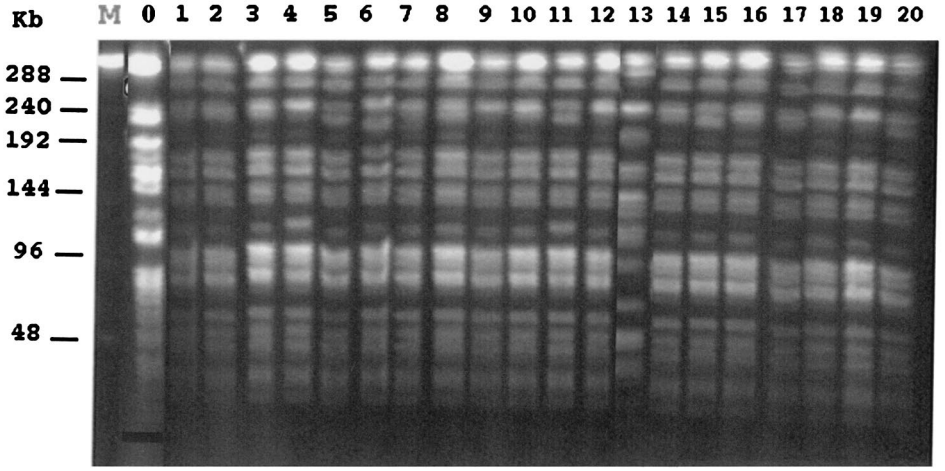

FIG. 4. PFGE profiles of *Apa*I-digested whole-cell DNA of 21 *A. baumannii* isolates. Lane 0, *A. baumannii* reference strain CIP7034^T; lanes 1 to 20, *A. baumannii* isolates 1 to 20 (clinical isolates 1 to 13 and environmental isolates 14 to 20, respectively). Clinical isolate 3 is also referred as AYE throughout the text. Lane M, bacteriophage lambda DNA ladder.

nii are difficult to identify and control in hospital settings, since this bacterial species may survive on dry surfaces for a long time (2).

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