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Molecular Epidemiology of Multidrug-Resistant *Acinetobacter baumannii* in a Tertiary Care Hospital in Naples, Italy, Shows the Emergence of a Novel Epidemic Clone▽

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The molecular epidemiology of multidrug-resistant *Acinetobacter baumannii* was investigated in two intensive care units of the V. Monaldi university hospital in Naples, Italy, from May 2006 to December 2007. Genotype analysis by pulsed-field gel electrophoresis (PFGE), trilocus sequence-based typing (3LST), and multilocus sequence typing (MLST) of *A. baumannii* isolates from 71 patients identified two distinct genotypes, one assigned to PFGE group A, 3LST group 1, and ST2 in 14 patients and the other to PFGE group B, 3LST group 6, and ST78 in 71 patients, that we named ST2/A and ST78/B, respectively. Of these, ST2/A corresponded to European clone II identified in the same hospital during 2003 and 2004; ST78/B was a novel genotype that was isolated for the first time in May 2006 but became prevalent during 2007. The ST78/B profile was also identified in five patients from two additional hospitals in Naples during 2007. The ST2/A and ST78/B isolates were resistant to all antimicrobials tested, including carbapenems, but were susceptible to colistin. Both ST2/A and ST78/B isolates possessed a plasmid-borne carbapenem-hydrolyzing oxacillinase gene, bla_{OXA-58}, flanked by IS*Aba2* and IS*Aba3* elements at the 5′ and 3′ ends, respectively. The selection of the novel ST78/B *A. baumannii* clone might have been favored by the acquisition of the bla_{OXA-58} gene.

Acinetobacter baumannii is an emerging opportunistic nosocomial pathogen, with increasing prevalence worldwide, responsible for a variety of nosocomial infections, especially in intensive care unit (ICU) patients (10, 15). Several hospital outbreaks caused by the selection of multiresistant *A. baumannii* clones have been described in Europe and worldwide (1, 10, 15, 27). Genotypic characterization of epidemic *A. baumannii* isolates through amplified fragment length polymorphism analysis has identified clusters of highly similar strains, which were assumed to represent distinct clonal lineages and were defined as European clones I, II, and III (9, 24). Similarly, three distinct groups were recently identified among *A. baumannii* isolates from five different countries by sequence-based typing (ST): group 1, corresponding to European clone II; group 2, corresponding to European clone I; and group 3, corresponding to European clone III (22). Moreover, epidemics caused by *A. baumannii* genotypes assigned to novel ST groups 4 and 5 have been recently described in different Greek and Turkish cities (11). The majority of the outbreaks occurring in Europe were caused by carbapenem-resistant strains that carried the bla_{OXA-58} gene or a distinct carbapenem-hydrolyzing oxacillinase (CHDL) gene (4, 8, 11–13, 16, 20, 27, 28).

We have previously reported the occurrence of two sequential outbreaks from August 1999 to February 2001 and from January 2002 to December 2002 along with the emergence of carbapenem-resistant *A. baumannii* in the ICU of the Federico II university hospitals in Naples, Italy, during 2002 (26). More recently, we have shown that the same epidemic *A. baumannii* clone isolated during 2002 was responsible for a large and sustained outbreak in the V. Monaldi tertiary care teaching hospital of Naples between June 2003 and June 2004 (25). An increase in the number of cases of *A. baumannii* infection was observed after 2 years in the V. Monaldi hospital. The objectives of the present study were (i) to investigate the molecular epidemiology of *A. baumannii* in the V. Monaldi hospital, (ii) to study the genetic characteristics of *A. baumannii* isolates responsible for the epidemic, and (iii) to analyze the antimicrobial susceptibilities of the *A. baumannii* isolates and their mechanisms of resistance.

MATERIALS AND METHODS

Setting and study period. The V. Monaldi hospital is a 600-bed tertiary care teaching hospital serving approximately 20,000 admissions per year. The hospital is provided with five ICUs: a neonatal ICU, a coronary ICU, a cardiac surgery ICU, a general and specialist surgery ICU (namely, a postoperative ICU [PO-ICU]), and a cardiorespiratory ICU (CR-ICU). The PO-ICU and CR-ICU are located in a recently renovated area of the hospital and are connected by a short internal corridor, and each has eight beds and an isolation box. Although spatially very close, the two wards have distinct staffs and medical equipment. Patients admitted to the PO-ICU are inpatients undergoing major elective surgery, while the CR-ICU admits both inpatients requiring intensive care and outpatients from other municipal or regional ICUs. The present study analyzed

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all 71 available *A. baumannii* isolates from 71 patients in the CR-ICU and PO-ICU wards between May 2006 and December 2007.

Microbiological surveillance and epidemiological data. Patient microbiological screening is routinely performed at admission to the CR-ICU and PO-ICU; further specimens are collected during patients' stays upon clinical judgment. Moreover, starting from January 2007 monthly reporting to the local infection control team of all microbiological isolations in high-risk areas was implemented. Analysis of data for the first part of the study period (May 2006 to December 2006) was performed retrospectively. Epidemiological data for the 76 *A. baumannii*-positive patients in the V. Monaldi, Cotugno, and A. Cardarelli hospitals (age, gender, primary diagnosis, infectious comorbidities, and outcome) were retrospectively collected from hospital discharge cards. *A. baumannii*-associated mortality was defined as death occurring during *A. baumannii* infection. Epidemiological and microbiological data were analyzed using SPSS v.11.0 (SPSS Inc., Chicago, IL) by means of Student's *t* test or Pearson's chi-square test as appropriate. Results were considered to be statistically significant at $P < 0.05$.

Bacterial strains and microbiological methods. *A. baumannii* isolates were obtained from clinical specimens by standard methods, followed by isolation in pure culture on MacConkey agar plates, and were stored at −80°C in nutrient broth containing 20% (vol/vol) glycerol. Strains were originally identified as *Acinetobacter baumannii-A. calcoaceticus* complex by using the Vitek 2 automatic system with the ID-GNB card for identification of Gram-negative bacilli (bioMerieux, Marcy-l'Etoile, France). *A. baumannii* species identification was confirmed by amplification of the bla_{OXA-51}-like gene and PCR amplification and sequence analysis of the 16S-23S rRNA intergenic spacer region (6, 22).

Antimicrobial susceptibilities. MICs were determined by a microdilution method according to Clinical and Laboratory Standards Institute document M7-A6 (7). Breakpoint values were those recommended by the CLSI (7). Breakpoints for colistin were those from the British Society for Antimicrobial Chemotherapy (BSAC) (5). Etest MBL strips (AB Biodisk, Solna, Sweden) were used to evaluate the presence of metallo-beta-lactamase (MBL) activity according to the manufacturer's procedure. The role of oxacillinase production in carbapenem resistance was assessed by determining carbapenem MICs by microdilution in the presence and absence of 200 mM NaCl, as described previously (28).

PFGE and dendrogram analysis. ApaI DNA macrorestriction and pulsed-field gel electrophoresis (PFGE) and dendrogram analysis of *A. baumannii* isolates were performed as previously reported (25). Because *A. baumannii* isolates were epidemiologically related, interpretation of genomic relatedness was performed using the criteria of Tenover et al. (19).

Identification of PCR-based sequence groups and ST. Multiplex PCRs and sequence-based typing (ST) were performed as previously described (22). Assignment of novel alleles and ST types was performed using the bioinformatic tools at the Health Protection Agency website on *A. baumannii* sequence typing that has been developed and maintained by J. F. Turton and R. Meyers (http://www.hpa-bioinformatics.org.uk/AB/home.php).

MLST. Multilocus sequence typing (MLST) analysis was performed using the Institut Pasteur's MLST scheme, publicly available from the MLST website at http://www.pasteur.fr/mlst. This MLST scheme is based on sequencing of an internal portion of the seven genes encoding 60-kDa chaperonin (*cpn60*), protein elongation factor EF-G (*fusA*), citrate synthase (*gltA*), CTP synthase (*pyrG*), homologous recombination factor (*recA*), 50S ribosomal protein L2 (*rplB*), and RNA polymerase subunit B (*rpoB*). Primer pairs for three of these genes (*cpn60*, *gltA*, and *recA*) were previously designed by Bartual et al. (3). Primer pairs for three other genes (*fusA*, *pyrG*, and *rplB*) are derived from primers initially proposed by Santos and Ochman (18). Finally, primers for gene *rpoB* were designed previously (17). PCR conditions were 35 cycles (denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min) preceded by a 3-min denaturation at 94°C and followed by a 5-min extension at 72°C. Further details on this MLST scheme can be found at www.pasteur.fr/mlst.

PCR analysis of carbapenemase genes and ISs. PCR analysis for carbapenemase-encoding genes in *Acinetobacter* spp. (bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{OXA-23}-like, bla_{OXA-24}-like, bla_{OXA-51}-like, and bla_{OXA-58}) was performed as previously described (28). PCR characterization of the bla_{OXA-58}-surrounding insertion sequence (IS) was performed as described previously (16). The colinearity between IS elements and the bla_{OXA-66} or bla_{OXA-90} gene was analyzed using primers for IS elements described previously by Poirel and Nordmann (16) and for the bla_{OXA-51}-like gene described previously by Turton et al. (23).

Plasmid analysis. Plasmid DNA preparations were performed by using the QIAfilter plasmid purification maxikit adapted for low-copy-number plasmids (Qiagen Corporation, Milan, Italy) according to the manufacturer's procedure. HindIII-generated fragments were separated on 1% agarose gels, transferred onto nylon membranes, and hybridized with PCR-generated probes specific for bla_{OXA-58}.

Mating experiments. Filter mating was performed using *A. baumannii* isolates of PFGE type A or B, resistant to imipenem and susceptible to rifampin, and *Acinetobacter* genomic species 3 strain 4442 (13), susceptible to imipenem but resistant to rifampin, as donor and recipient cells, respectively. Transconjugants were selected on brain heart infusion (BHI) agar plates containing imipenem (16 mg/liter) plus rifampin (100 mg/liter). The frequency of transfer was calculated as the number of transconjugants divided by the number of surviving recipients.

DNA sequencing and computer analysis of sequencing data. DNA sequences of plasmid pABNA1 and pABNA2 were amplified using primers 5′-GTCACG CCAGTATTAACCAA-3′ and 5′-TCGTTTACCCCAAACATAAGC-3′, spanning plasmid *oriV* and IS*Aba3*, respectively. DNA sequencing of PCR products was performed using the ABI Prism BigDye Terminator v3.1 ready reaction cycle sequencing kit and the 3730 DNA analyzer (Applied Biosystems, Foster City, CA). DNA sequences were assembled using the program Autoassembler version 1.4 (Applied Biosystems, Foster City, CA) and annotated using the BLAST program (2) and the sequence annotation tools integrated into the Sequin program version 7.9 available at http://www.ncbi.nlm.nih.gov/Sequin/index.html.

Nucleotide sequence accession numbers. The nucleotide sequences of the novel alleles identified for *A. baumannii* isolates of ST group 6 have been deposited in the GenBank nucleotide database under accession numbers EU433384 (*ompA* allele 7), EU433383 (*csuE* allele 10), and EU433382 (bla_{OXA-51}-like allele 9 corresponding to bla_{OXA-90}). The nucleotide plasmid sequences pABNA1and pABNA2, amplified from isolates 3979 (PFGE type A) and 3957 (PFGE type B), have been assigned accession numbers GQ338082 and GQ338083, respectively, in the GenBank nucleotide database. Allele sequences of ST2 and ST78 are available from the Institut Pasteur's *A. baumannii* MLST website at www.pasteur.fr/mlst.

RESULTS

Molecular epidemiology of *A. baumannii* in the hospital. We have recently described an *A. baumannii* outbreak between June 2003 and June 2004 in the V. Monaldi hospital of Naples, Italy (25). During the two subsequent years, only a few sporadic cases of *A. baumannii* infection were detected in the hospital. The epidemiology of *A. baumannii* was studied in the CR-ICU and PO-ICU of the hospital between May 2006 and December 2007, when a further increase of the number of *A. baumannii* isolates was observed in the two wards, while no isolates were obtained from other wards. During the study period, a total of 1,760 patients were admitted to the two wards (514 and 1,240 to the CR-ICU and PO-ICU, respectively). *A. baumannii* was isolated from 101 patients, with an overall *A. baumannii* isolation rate of 5.7% (14.6% and 2.1% for the CR-ICU and PO-ICU, respectively; $P < 0.05$). *A. baumannii* was isolated in 75 CR-ICU patients who showed a mean number of positive specimens of 2.17 ± 1.58. In the PO-ICU 26 patients proved to be positive for *A. baumannii*, with a mean number of positive specimens of 2.08 ± 1.35 ($P > 0.05$). Mean length of stay in the two wards was calculated and proved to be 10.98 ± 22.32 and 2.22 ± 2.94 days for the CR-ICU and PO-ICU, respectively ($P < 0.05$).

To investigate whether the increase in the frequency of isolation of *A. baumannii* during the study period was caused by the spread of epidemic clones, all 71 available, nonrepetitive *A. baumannii* isolates from 71 patients between May 2006 and December 2007 were genotyped: 54 were from CR-ICU patients (73.3% of patients) and 17 were from PO-ICU patients (61.5% of patients). One *Acinetobacter* isolate was not included in the study because its species identification as *A. baumannii* was not confirmed by molecular methods. Molecular typing by PFGE identified two major PFGE groups, which differed in

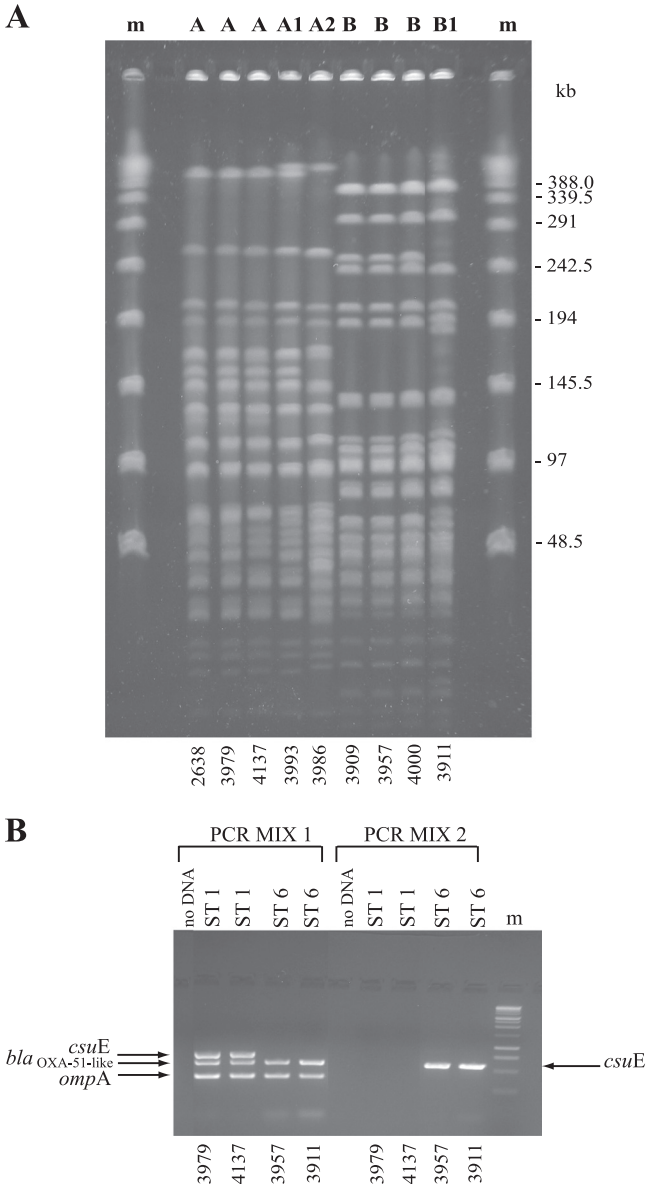

FIG. 1. (A) ApaI PFGE profiles of representative *A. baumannii* strains included in the study. Capital letters above the lanes indicate PFGE types identified; m, phage lambda DNA molecular mass markers. The isolate number is shown below each lane. Sizes of lambda DNA molecular mass markers are shown on the right. (B) Multiplex PCR to selectively amplify *ompA*, *csuE*, and $bla_{OXA\text{-}51}$-like alleles. ST groups identified are indicated above the lanes; m, 1-kb DNA ladder molecular mass markers (Promega, Milan, Italy). The isolate number is shown below each lane.

the migration of more than six bands, in 14 and 57 isolates, which we named A and B, respectively. Of the 14 isolates of PFGE group A, 12 showed an identical macrorestriction pattern (type A) whereas two differed in the migration of at least two and three DNA fragments and were classified into subtypes A1 and A2, respectively. Fifty-six isolates of PFGE group B showed an identical macrorestriction pattern (type B),whereas one differed in the migration of up to six bands and was designated subtype B1 (Fig. 1A and Table 1). The epidemic PFGE profile A was identical to that of the epidemic *A. baumannii* strain 2638 isolated in the V. Monaldi hospital in 2004 (25) (Fig. 1A). Genotype analysis using the multiplex PCRs and trilocus sequence-based typing (3LST) approach described by Turton et al. (22) assigned all 14 isolates of PFGE group A to previously defined three-locus sequence type group 1 (Fig. 1B and Table 1). The multiplex PCR approach identified a distinct PCR pattern in the other 57 isolates of PFGE group B with the amplification of $bla_{OXA\text{-}51}$-like and *ompA* but not *csuE* alleles in PCR mix 1 and amplification of *csuE* but not $bla_{OXA\text{-}51}$-like and *ompA* alleles in PCR mix 2 (Fig. 1B). Trilocus sequence-based typing identified an identical allele profile, 7/10/9, at *ompA*/*csuE*/$bla_{OXA\text{-}51}$-like loci in the 57 isolates of PFGE group B that were assigned to novel 3LST group 6 (Table 1). The differences in group-specific PCR patterns facilitated rapid identification of the *A. baumannii* isolates belonging to different ST groups, 1 and 6, in the hospital and were used as a preliminary typing approach for the isolates. MLST based on the conserved regions of *cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB* housekeeping genes identified ST2 for isolates of PFGE type A and subtypes A1 and A2 (group A), allelic profile 25/3/6/2/28/1/29, which corresponds to a novel ST assigned as ST78 for isolates of PFGE type B and subtype B1 (group B), respectively (Table 1). Based on all the above data, two distinct genotypes were identified, one assigned to PFGE group A (including PFGE type A and subtypes A1 and A2), 3LST group 1, and ST2, and the other assigned to PFGE group B (which included PFGE type B and subtype B1), 3LST group 6, and ST78, which we named ST2/A and ST78/B, respectively. The differences in PFGE patterns observed between subtypes A1 and A2 and type A and between subtype B1 and type B were probably due to the higher discriminatory power of PFGE than of MLST and were not further considered.

Molecular epidemiology of *A. baumannii* isolates showed that the outbreak in the two wards of the V. Monaldi hospital was caused by the spread of ST2/A and ST78/B genotypes, which were isolated in two consecutive temporal clusters, one clone predominating over the other. In fact, ST2/A was identified in 10 CR-ICU patients and in 4 PO-ICU patients, while ST78/B was found in 44 CR-ICU patients and in 13 PO-ICU patients. Moreover, ST2/A isolates occurred between May 2006 and February 2007 in both wards, while ST78/B isolates were first identified in the CR-ICU ward in May 2006 and predominated in both wards from March 2007 onward (Fig. 2). The lower respiratory tract was the most frequent site of isolation (9 of 14 and 45 of 57 patients for ST2/A and ST78/B, respectively) ($P > 0.05$) and was associated with clinical infection as the primary diagnosis or infectious comorbidity in 3 of 10 and 11 of 44 patients for ST2/A and ST78/B, respectively ($P > 0.05$). Nine *A. baumannii* isolates from blood were assigned to ST78/B, and one was assigned to ST2/A ($P > 0.05$), and they were always associated with clinical infection. ST78/B was also isolated from one wound swab, the urinary tract of one patient, and one catheter tip, while ST2/A was also isolated from the upper respiratory tract of one patient and the central venous catheter and the catheter tip of two patients and one patient, respectively. The mean ages of patients infected by ST2/A and ST78/B were 75.5 ± 9.84 and 59.79 ± 14.42 years, respectively ($P > 0.05$). The female/male ratios for patients

TABLE 1. Epidemiological, phenotypic and genotypic features of *A. baumannii* strains included in the study[a]

Hospital	Strain	Culture date (day/mo/yr)	Ward	Age (yr)/ gender	Primary diagnosis	Infectious comorbidity	Outcome	Isolate source	PFGE type	3LST group	MLST ST	MIC (mg/liter) of antibiotic:						
												IPM	MEM	SAM	AK	GM	RIF	COL
Monaldi	3978	15/05/2006	CR-ICU	61/M	Respiratory failure	Pneumonia	E	BA	B	6	78	32	16	125	125	32	4	≤0.5
	3979	29/05/2006	CR-ICU	85/F	Respiratory failure	Pneumonia	VD	BA	A	1	2	32	8	32	125	8	4	≤0.5
	3980	05/06/2006	CR-ICU	67/F	Respiratory failure	Pneumonia*	E	BA	B	6	78	16	8	125	125	32	4	≤0.5
	3982	09/06/2006	PO-ICU	81/F	Acute myocardial infarction		E	BA	A	1	2	16	16	32	125	8	2	≤0.5
	3993	19/06/2006	CR-ICU	83/F	Pneumonia		E	BA	A1	1	2	16	4	32	125	8	2	≤0.5
	3983	28/06/2006	CR-ICU	67/M	Lung cancer	Sepsis	E	BC	A	1	2	16	4	125	4	8	>500	≤0.5
	3991	17/07/2006	CR-ICU	79/F	Pneumonia*		T	PS	A	1	2	16	4	32	4	8	4	≤0.5
	3994	17/07/2006	CR-ICU	75/M	Aortic dissection		E	BA	B	6	78	8	4	125	125	32	2	≤0.5
	3984	17/07/2006	CR-ICU	81/F	Respiratory failure		D	BA	A	1	2	16	8	32	32	8	4	≤0.5
	4137	31/07/2006	CR-ICU	67/F	Respiratory failure	Pneumonia	E	BA	A	1	2	8	4	125	125	256	>500	≤0.5
	3987	03/08/2006	CR-ICU	47/F	Respiratory failure	Pneumonia	E	BA	B	6	78	16	4	64	125	32	4	≤0.5
	3985	14/08/2006	CR-ICU	67/M	Pneumonia		E	BA	B	6	78	16	16	125	16	4	2	1
	3992	21/08/2006	CR-ICU	50/F	Respiratory failure		E	BA	A	1	2	32	8	32	125	8	4	≤0.5
	3990	23/08/2006	CR-ICU	76/M	Pneumonia		T	CVC	A	1	2	32	16	64	16	4	>500	≤0.5
	3989	28/08/2006	CR-ICU	59/M	Esophageal cancer	Pneumonia*	E	BA	B	6	78	16	8	125	125	256	2	1
	4138	12/09/2006	PO-ICU	68/M	Lung cancer		E	BA	A	1	2	8	8	32	16	4	>500	≤0.5
	3986	06/11/2006	CR-ICU	19/M	Respiratory failure		E	CT	A2	1	2	16	4	32	>250	8	4	≤0.5
	3988	06/11/2006	CR-ICU	76/F	Respiratory failure		D	BA	A	1	2	32	4	32	125	8	4	≤0.5
	3956	05/01/2007	PO-ICU	26/M	Respiratory failure	Sepsis	E	BC	B	6	78	32	8	64	125	32	4	≤0.5
	4155	08/01/2007	PO-ICU	81/M	Abdominal aortic aneurysm	Vascular prosthesis infection*	E	BA	A	1	2	16	16	125	16	64	>500	≤0.5
	4156	08/01/2007	PO-ICU	68/M	Larynx cancer		D	BA	B	6	78	8	8	125	>250	8	6	≤0.5
	3958	15/01/2007	CR-ICU	81/M	Respiratory failure		E	BA	B	6	78	32	8	125	32	32	16	≤0.5
	4157	15/01/2007	PO-ICU	66/M	Colorectal cancer		VD	BA	B	6	78	16	8	125	>250	32	4	≤0.5
	3957	18/01/2007	PO-ICU	44/M	Bronchiectasis	Lung abscess	D	BA	B	6	78	16	8	125	32	32	4	≤0.5
	3942	24/01/2007	PO-ICU	81/F	Respiratory failure		VD	BA	B	6	78	16	8	125	125	32	16	≤0.5
	3997	29/01/2007	CR-ICU	85/F	Respiratory failure		E	BA	B	6	78	16	8	64	16	32	2	≤0.5
	4158	03/02/2007	PO-ICU	56/M	Lung cancer		E	CVC	A	1	2	16	16	64	>250	8	>500	≤0.5
	3944	12/02/2007	PO-ICU	65/F	Intestinal occlusion		E	BA	B	6	78	16	8	125	>250	32	4	≤0.5
	3945	16/02/2007	PO-ICU	39/M	Aortic dissection		T	BA	B	6	78	8	8	64	32	64	4	≤0.5
	3960	23/02/2007	CR-ICU	45/M	Pneumocystosis*	Sepsis	E	BC	B	6	78	16	8	125	32	32	2	≤0.5
	3998	03/03/2007	CR-ICU	52/F	Respiratory failure	Sepsis	E	BC	B	6	78	16	8	125	>250	32	4	≤0.5
	3946	13/03/2007	PO-ICU	75/M	Unspecified hemorrhage		E	WS	B	6	78	8	8	125	>250	32	4	≤0.5
	3999	19/03/2007	CR-ICU	66/F	Mediastinitis		E	BA	B	6	78	8	4	64	125	32	4	≤0.5
	3961	21/03/2007	CR-ICU	77/M	Aortic dissection		E	BA	B	6	78	8	8	125	125	64	2	≤0.5
	4000	21/03/2007	CR-ICU	62/M	Respiratory failure		E	BA	B	6	78	16	16	125	125	32	4	≤0.5
	3962	10/04/2007	CR-ICU	71/F	Respiratory failure		E	BA	B	6	78	32	4	125	32	32	2	≤0.5
	4001	30/04/2007	CR-ICU	78/M	Respiratory failure		D	BA	B	6	78	32	8	125	125	32	4	≤0.5
	4002	30/04/2007	CR-ICU	75/F	Pneumonia		E	BA	B	6	78	8	4	125	32	64	4	≤0.5
	3963	04/05/2007	CR-ICU	76/F	Respiratory failure		E	BA	B	6	78	16	4	125	32	32	4	≤0.5
	3947	08/05/2007	PO-ICU	75/M	Bladder cancer		D	UC	B	6	78	16	4	125	125	32	4	≤0.5
	4139	14/05/2007	CR-ICU	57/F	Respiratory failure		E	CT	B	6	78	16	8	64	125	32	4	≤0.5
	3948	26/05/2007	PO-ICU	64/M	Colorectal cancer	Sepsis	E	BC	B	6	78	8	8	125	125	32	4	≤0.5
	3969	04/06/2007	CR-ICU	74/M	Wegener's granulomatosis		E	BA	B	6	78	8	8	64	16	32	4	≤0.5
	4140	05/06/2007	CR-ICU	79/F	Respiratory failure		E	BA	B	6	78	8	8	125	125	32	4	≤0.5
	3970	19/06/2007	CR-ICU	52/F	Acute myocardial infarction	Pneumonia	E	BA	B	6	78	16	8	64	16	64	4	≤0.5
	4141	21/06/2007	CR-ICU	49/M	Respiratory failure		T	BA	B	6	78	16	4	64	16	32	4	≤0.5
	3971	25/06/2007	CR-ICU	68/F	Aortic dissection	Sepsis	VD	BC	B	6	78	32	8	125	125	64	4	≤0.5
	4142	28/06/2007	CR-ICU	76/M	Respiratory failure		E	BA	B	6	78	8	4	125	16	32	4	≤0.5
	4143	02/07/2007	CR-ICU	54/M	Amyotrophic lateral sclerosis	Pneumonia	D	BA	B	6	78	8	8	125	125	32	4	≤0.5
	3972	16/07/2007	CR-ICU	85/M	Respiratory failure	Pneumonia	T	BA	B	6	78	8	8	125	>250	32	4	≤0.5
	4145	23/07/2007	CR-ICU	90/M	Acute myocardial infarction		E	BA	B	6	78	16	8	125	125	32	8	≤0.5
	4144	24/07/2007	CR-ICU	70/F	Respiratory failure		E	BA	B	6	78	8	8	64	16	32	4	≤0.5
	4136	30/07/2007	CR-ICU	84/F	Respiratory failure		E	BA	B	6	78	16	4	125	125	32	4	≤0.5
	3966	01/08/2007	CR-ICU	51/M	Renal ptosis	Sepsis	T	BC	B	6	78	32	8	125	125	32	4	≤0.5
	4146	02/08/2007	CR-ICU	53/F	Respiratory failure		E	BA	B	6	78	8	4	125	16	32	4	≤0.5
	4147	04/08/2007	CR-ICU	70/M	Respiratory failure		E	BA	B	6	78	32	16	125	125	32	4	≤0.5
	3967	03/09/2007	CR-ICU	77/F	Respiratory failure		T	BA	B	6	78	8	8	125	125	32	4	≤0.5
	4148	03/09/2007	CR-ICU	70/M	Respiratory failure		E	BA	B	6	78	16	8	125	>250	32	2	≤0.5
	4149	05/09/2007	CR-ICU	58/F	Respiratory failure	Pneumonia	E	BA	B	6	78	8	4	125	125	32	4	≤0.5
	4150	10/09/2007	CR-ICU	76/F	Respiratory failure		E	BA	B	6	78	16	8	125	125	32	4	≤0.5

3974	12/09/2007	CR-ICU	72/M	Respiratory failure	Sepsis	E	BC	B	6	78	8	4	125	16	32	4	≤0.5
4151	01/10/2007	CR-ICU	77/M	Respiratory failure		E	BA	B	6	78	16	16	64	125	32	4	≤0.5
4152	12/10/2007	CR-ICU	63/M	Respiratory failure		E	BA	B	6	78	8	4	125	>250	8	4	≤0.5
3950	12/10/2007	PO-ICU	72/M	Respiratory failure		E	BA	B	6	78	8	4	64	16	32	4	≤0.5
3911	24/10/2007	CR-ICU	74/M	Respiratory failure	Sepsis	VD	BC	B1	6	78	32	8	125	>250	64	4	≤0.5
4153	03/11/2007	CR-ICU	69/F	Respiratory failure		E	BA	B	6	78	8	8	125	125	64	4	≤0.5
3909	05/11/2007	CR-ICU	44/F	Respiratory failure	Pneumonia*	E	BA	B	6	78	32	8	125	2	32	4	≤0.5
3951	14/11/2007	PO-ICU	49/M	Pancreatic cancer		E	BA	B	6	78	16	8	125	2	256	2	≤0.5
4154	19/11/2007	CR-ICU	62/M	Respiratory failure	Pneumonia	E	BAL	B	6	78	16	8	125	2	32	4	≤0.5
3952	24/11/2007	PO-ICU	79/M	Abdominal aortic aneurysm	Sepsis	E	BC	B	6	78	16	8	125	2	32	4	≤0.5
3912	22/10/2007	CR-ICU	45/M	Acute myocardial infarction		E	BA	B	6	78	8	8	125	>250	32	4	≤0.5
Cotugno 3678	07/04/2007	ICU	60/M	Respiratory failure	Pneumonia	D	BA	B	6	78	32	16	125	125	4	4	≤0.5
3679	24/05/2007	ICU	75/M	Cholangiocarcinoma	Sepsis	D	BC	B	6	78	16	8	125	125	32	4	≤0.5
3701	06/06/2007	ICU	60/F	Sepsis		E	BC	B	6	78	16	8	125	125	32	4	≤0.5
3696	03/10/2007	ICU	60/F	Tetanus*		D	CVC	B	6	78	16	8	125	>250	64	4	≤0.5
Cardarelli 3933	04/02/2007	ICU	19/M	Polytrauma	Sepsis	D	BC	B	6	78	1	0.5	125	32	32	4	≤0.5

[a] Abbreviations: CR-ICU, cardiorespiratory intensive care unit; PO-ICU, postoperative intensive care unit; ICU, intensive care unit; F, female; M, male; PFGE, pulsed-field gel electrophoresis; 3LST, trilocus sequence-based typing; MLST, multilocus sequence typing; ST, sequence type; E, exitus; VD, voluntary discharge; T, transferred; D, discharge; BA, bronchial aspirate; BAL, bronchoalveolar lavage; BC, blood culture; CVC, central venous catheter; CT, catheter tip; UC, urine culture; PS, pharyngeal swab; WS, wound swab; IPM, imipenem; MEM, meropenem; SAM, sulbactam-ampicillin; AK, amikacin; GM, gentamicin; RIF, rifampin; COL, colistin. Infections not attributable to *A. baumannii* are indicated by an asterisk.

FIG. 2. Molecular epidemiology of *A. baumannii* in the V. Monaldi hospital, Naples, Italy, during 2006 and 2007. Gray and white bars represent ST2/A isolates from the cardiorespiratory ICU (CR-ICU) and postoperative ICU (PO-ICU), respectively; black and dotted bars represent ST78/B isolates from the CR-ICU and PO-ICU, respectively.

infected by ST2/A and ST78/B were 3:1 and 0.46:1, respectively ($P > 0.05$). Crude mortality and *A. baumannii*-associated mortality were 64% (9/14) and 21% (3/14), respectively, for patients with isolation of ST2/A *A. baumannii* and 75% (43/57) and 25% (14/57), respectively, for patients with isolation of ST78/B *A. baumannii* (Table 1).

Five additional multidrug-resistant (MDR) *A. baumannii* strains with the ST78/B profile were isolated in the ICU wards of the Cotugno and A. Cardarelli hospitals in Naples (4 isolates and 1 isolate, respectively) during 2007 (Table 1).

Antimicrobial susceptibility patterns of *A. baumannii* isolates. Both ST2/A and ST78/B *A. baumannii* isolates from the V. Monaldi hospital showed a multidrug-resistant antibiotype. In particular, they were resistant to ampicillin-sulbactam, piperacillin-tazobactam, broad-spectrum cephalosporins, fluoroquinolones, and aminoglycosides; intermediate or resistant to imipenem and rifampin; and intermediate to meropenem but were susceptible to colistin sulfate (Table 2). Interestingly, 5 isolates with the ST2/A profile showed high-level resistance to rifampin (Tables 1 and 2). All four *A. baumannii* isolates with the ST78/B profile from the Cotugno hospital in Naples showed antimicrobial susceptibility profiles identical to those of ST78/B isolates from the V. Monaldi hospital; the single *A. baumannii* ST78/B isolate from the A. Cardarelli hospital was susceptible to imipenem (MIC, 1.0 mg/liter) and meropenem (MIC, 0.5 mg/liter) (Table 1). Tests with Etest MBL strips showed that all carbapenem-resistant ST2/A and ST78/B isolates were intermediate or resistant to imipenem (MICs, 8 to 16 mg/liter) but were negative for MBL production (imipenem-EDTA MICs, 4 to 8 mg/liter). To study the contribution of oxacillinases to imipenem resistance, imipenem MICs were analyzed in the presence of 200 mM NaCl for carbapenem-resistant ST2/A and ST78/B isolates by a microdilution method. These experiments showed that imipenem MICs (16 mg/liter) were inhibited by up to 8-fold in the presence of NaCl (2 mg/liter).

TABLE 2. Antibiotic susceptibility profiles of *A. baumannii* isolates from the V. Monaldi hospital[a]

Antibiotic	MIC (mg/liter)					
	ST2/A (14 total strains)			ST78/B (57 total strains)		
	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range
Sulbactam-ampicillin	32	125	32–125	125	125	64–125
Piperacillin-tazobactam	125	250	125–>250	250	250	32–>250
Ceftazidime	250	250	125–>250	250	250	125–>250
Cefepime	125	250	16–250	16	32	16–250
Imipenem	16	32	8–32	16	32	8–32
Meropenem	8	16	4–16	8	16	4–16
Amikacin	125	>250	4–>250	125	>250	2–>250
Gentamicin	8	64	4–>250	32	64	4–>250
Ciprofloxacin	64	250	32–>250	64	250	32–>250
Rifampin	4	500	2–500	4	4	2–16
Colistin	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5–1

[a] *A. baumannii* isolates were analyzed by a microdilution method for MIC determination according to CLSI guidelines.

Molecular analysis of carbapenem resistance in *A. baumannii* isolates. PCR and sequence analysis identified a bla_{OXA-58} gene flanked by IS*Aba2* and IS*Aba3* elements at the 5′ and 3′ ends, respectively, in plasmid DNA from all carbapenem-resistant *A. baumannii* ST2/A and ST78/B isolates but not from the single carbapenem-susceptible *A. baumannii* strain of PFGE type B isolated in the A. Cardarelli hospital. No amplification products were obtained from chromosomal or plasmid DNA of *A. baumannii* ST2/A and ST78/B isolates using primers for bla_{IMP}-type, bla_{VIM}-type, or bla_{SIM}-type MBLs or bla_{OXA-23} or $bla_{OXA-24/40}$ CHDLs. Also, PCR experiments failed to identify any IS element upstream of the naturally occurring bla_{OXA-66} or bla_{OXA-90} genes in *A. baumannii* ST2/A and ST78/B isolates, respectively, thus excluding the notion that IS-mediated overexpression of these oxacillinases may account for the resistance to imipenem (23).

Genetic location and characterization of the genetic structures surrounding the bla_{OXA-58} gene. Digestion of plasmid DNA from *A. baumannii* ST2/A and ST78/B isolates with HindIII enzyme revealed different restriction patterns that generated two different positive bands of approximately 3.0 and 2.7 kb and 2.7 and 1.0 kb, respectively, when hybridized with a bla_{OXA-58}-specific probe (Fig. 3A). The direct sequence of amplicons generated from plasmid DNA preparation of *A. baumannii* ST2/A and ST78/B isolates using primers spanning the 5′ end of *A. baumannii* origin of plasmid replication (*oriV*) and the 3′ end of the IS*Aba3* element identified two similar fragments of 6,095 and 6,073 bp that were designated pABNA1 and pABNA2, respectively.

The two amplicons showed identical origins of replication (*oriV*); a repeat region composed of five 22-bp-long imperfect direct iterons in pABNA1 and four 22-bp-long iterons in pABNA1 and pABNA2, respectively; identical *repAci1* and *repAci2* replicase genes; and a single copy of the bla_{OXA-58} gene that was flanked by IS*AbA2* and IS*Aba3* elements at the 5′ and 3′ ends, respectively (Fig. 3B). Filter mating experiments demonstrated that resistance to imipenem, along with the bla_{OXA-58} gene, was transferred from *A. baumannii* ST78/B isolate 3957, but not from *A. baumannii* ST2/A isolate 3979, to imipenem-susceptible *Acinetobacter* genomic species 3 isolate 4442 at a frequency of 1×10^{-6}. Imipenem MICs for transconjugants were similar (16 mg/liter) to those for donor isolates.

DISCUSSION

In the present report, we studied the molecular epidemiology and the genetic basis of carbapenem resistance in *A. baumannii* strains isolated between May 2006 and December 2007 during an epidemic occurring in two ICUs of the V. Monaldi hospital in Naples. In accordance with previous data (10, 15),

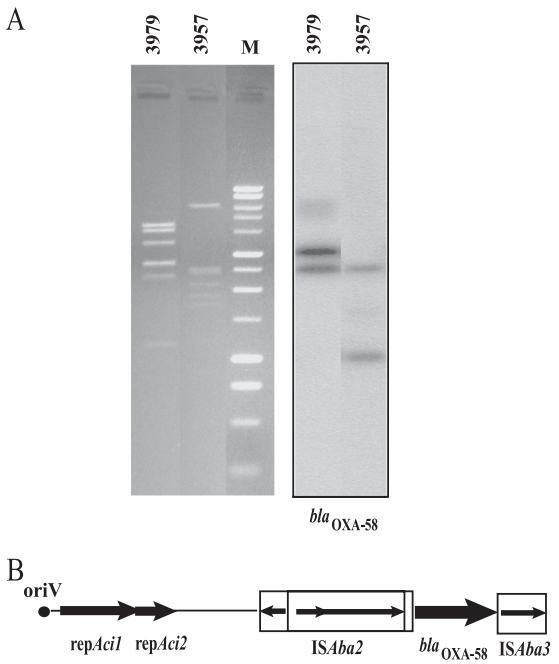

FIG. 3. (A) Plasmid localization of the bla_{OXA-58} gene in *A. baumannii* ST2/A and ST78/B isolates. Agarose (1%) gel electrophoresis in 1× Tris-acetate-EDTA buffer of HindIII-digested plasmids from *A. baumannii* isolates 3979 and 3957, respectively, stained with ethidium bromide and visualized under UV light and Southern blot hybridization with the bla_{OXA-58} probe are shown. Lane M shows a 1-kb DNA ladder (Promega, Milan, Italy). (B) Schematic map of the genetic structure surrounding the bla_{OXA-58} gene in *A. baumannii* ST2/A and ST78/B isolates. The genes and their corresponding transcription orientations are indicated by horizontal arrows. IS elements are represented by open rectangles filled with black arrows indicating the transposase gene and the direction of transcription. Names of relevant features are reported below or above the map.

the isolation rate of *A. baumannii* was significantly associated with length of stay in the ward, being higher in the CR-ICU than in the PO-ICU (10, 15). Based on a previous study of an *A. baumannii* outbreak occurring in the same institution between June 2003 and June 2004 (25), we could assume that the epidemic described herein was caused by the spread of a single epidemic clone. However, the present report revealed the emergence of two distinct *A. baumannii* epidemic clones that were isolated in two consecutive temporal clusters in the same wards of the hospital. Indeed, the identity of 3LST, ST, and resistance profiles/genes and the near-identity of PFGE profiles indicate that these two sets of isolates each represent a clone. The first epidemic clone showed identical PFGE profiles of the *A. baumannii* strains responsible for two epidemics occurring in Naples in the Federico II and V. Monaldi hospitals during 2002 and during 2003 and 2004, respectively (25, 26), and was assigned to 3LST group 1 and ST2, which corresponded to the previously characterized European clone II (9, 10). The second epidemic clone, which was first isolated in the CR-ICU in May 2006 and replaced the previous clone in both wards from March 2007 onward, showed a distinct genotype, assigned to novel 3LST group 6 and ST78, which has never been isolated before and is described for the first time herein. This is consistent with previous studies showing that carbapenem-resistant *A. baumannii* epidemics in southern Europe are caused by genotypes belonging to 3LST groups 1 and 2, corresponding to the European clones II and I, respectively, but also by additional genotypes of 3LST groups 4 and 5 (10, 11, 15, 22). Our data are also in agreement with a recent report showing that four distinct clones are responsible for a cluster of carbapenem-resistant *A. baumannii* infections in the ICU of a Greek hospital (21). Also, the isolation of *A. baumannii* ST78/B strains in the ICUs of two other hospitals in Naples during 2007 suggests that the spread of the novel *A. baumannii* epidemic clone described herein might have been caused by interhospital transfer of colonized patients in the city. In agreement with previous studies, the respiratory tract was the most frequent site of isolation for both clones (9, 15, 25, 26). However, ST78/B strains caused a higher but not statistically significant proportion of bacteremias than did the other clone in patients from the V. Monaldi hospital, thus suggesting that the novel epidemic clone may possess some inherent properties for developing invasive disease. Moreover, no statistically significant differences in mean age and female/male ratio were observed between patients of the V. Monaldi hospital infected by ST2/A strains and those infected by ST78/B strains of *A. baumannii*.

Several studies demonstrate that *A. baumannii* epidemic strains are selected in the hospital setting because of their multiple antimicrobial resistances (1, 9, 13, 15, 21, 27). In particular, the emergence of carbapenem resistance has been reported during hospital outbreaks of multidrug-resistant *A. baumannii* in Italy and southern Europe (4, 8, 11, 13, 20, 21, 25–28). Accordingly, the two *A. baumannii* clones described in the present study showed similar antibiotypes, characterized by resistance to all classes of antimicrobials, including carbapenems, and intermediate resistance to rifampin but susceptibility to colistin. Additional epidemiological information was provided by molecular analysis of carbapenem resistance genes. A plasmid-borne $bla_{\text{OXA-58}}$ gene was identified in both *A. baumannii* clones isolated in the V. Monaldi hospital but not in the single carbapenem-susceptible *A. baumannii* ST78/B isolate from the A. Cardarelli hospital. Although the plasmids carrying the $bla_{\text{OXA-58}}$ gene from the two epidemic clones showed distinct restriction patterns, two similar amplicons containing an origin of plasmid replication, a repeat region composed of four or five 22-bp imperfect direct iterons, the replicase genes, and a single copy of the $bla_{\text{OXA-58}}$ gene flanked by IS*Aba2* and IS*Aba3* sequences at the 5′ and 3′ ends of the gene, respectively, were identified. The above genetic structures were highly homologous with those found in plasmids pOUR and pACICU1 from *A. baumannii* strains 183 and ACICU, respectively, isolated in Rome, Italy (4, 14). Interestingly, all *A. baumannii* strains carrying the $bla_{\text{OXA-58}}$ gene isolated in Rome were assigned to ST group 1 and European clone II (4, 8, 14), as were the *A. baumannii* strains responsible for the outbreak occurring in the V. Monaldi hospital during 2003 and 2004 (11, 25). A $bla_{\text{OXA-58}}$ gene flanked by IS*Aba2* and IS*Aba3* sequences has been also found in plasmids isolated in strains from France and Spain showing distinct pulsotypes (16) and in plasmids isolated in strains from Greece assigned to ST groups 1 and 2 (11). The above data all suggest that carbapenem resistance in the two *A. baumannii* epidemic clones might have been acquired through horizontal gene transfer among distinct clones. Because clone ST2/A carrying a plasmid-borne $bla_{\text{OXA-58}}$ gene was first isolated in the V. Monaldi hospital during 2003 (25) while the first isolation of clone ST78/B carrying a plasmid-borne $bla_{\text{OXA-58}}$ gene occurred during 2006 in the hospital and one carbapenem-susceptible *A. baumannii* strain with the ST78/B profile was isolated in another hospital of Naples during 2007, we can make the hypothesis that plasmid sequences carrying the $bla_{\text{OXA-58}}$ gene flanked by IS*Aba2* and IS*Aba3* elements were transferred from ST2/A strains to ST78/B strains. In further support of this, we demonstrated herein that resistance to imipenem, along with the $bla_{\text{OXA-58}}$ gene, was transferred from ST78/B strains into the imipenem-susceptible *Acinetobacter* genomic species 3 strain.

In conclusion, molecular epidemiology of *A. baumannii* in the V. Monaldi hospital showed the occurrence of a novel epidemic clone that successfully spread among different wards and was selected because of the presence of a plasmid-borne $bla_{\text{OXA-58}}$ gene. This emphasizes the need to study the global epidemiology of *A. baumannii* and its associated antimicrobial resistances by using molecular typing methods in order to control the epidemic spread of multidrug-resistant *A. baumannii* infections in the hospital setting.

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