Outbreak of *Acinetobacter baumannii* Producing the Carbapenem-Hydrolyzing Oxacillinase OXA-58 in Rome, Italy

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ABSTRACT

In this study 45 epidemic and sporadic isolates of *Acinetobacter baumannii* were investigated by antimicrobial resistance, integron identifications and genotyping. Isolates were genotyped by random amplified polymorphism (RAPD) DNA and pulsed-field gel electrophoresis (PFGE). Four different RAPD patterns were observed among the isolates of our collection, further discerned in six PFGE types. Two prevalent genotypes were identified, one corresponding to a carbapenem resistant epidemic clone, causing an outbreak at the intensive care unit of a hospital of Rome. Two class 1 integrons, carrying different gene cassette arrays, were identified among the two prevalent genotypes. Nucleotide analysis of the integron-variable regions revealed the presence of the *aacA4*, *orfO*, *bla_{OXA-20}*, and *aacC1*, *orfX*, *orfX'*, *aadA1* gene cassette arrays, respectively. All the carbapenem resistant strains analyzed in this study carried the *bla_{OXA-58}* gene located on plasmids.

INTRODUCTION

ACINETOBACTER BAUMANNII is an important opportunistic pathogen responsible for nosocomial infections. While carbapenems are the drugs of choice against *Acinetobacter* infections, carbapenem resistance is now becoming common in this species.^{6,7,10,16,21,28,32,37} *Acinetobacter* may develop resistance to carbapenems through various mechanisms including decreased permeability, efflux pump overexpression, and production of carbapenemases.^{24,26,36} Four of the eight carbapenemase-hydrolyzing oxacillinase (OXA) clusters have been identified in *A. baumannii*, including the OXA-23, OXA-24, OXA-51, and OXA-58 enzymes and their variants,^{15,25,27} but the emergence of the OXA-58, first reported in a *A. baumannii* isolate in 2003 in France,²⁵ has been recently observed in many different collections of strains isolated worldwide.^{5,20} A preliminary study, performed in Italy on eight sporadic *A. baumannii* isolates, recovered from July to September 2005 from patients of the Policlinico Umberto I, the largest university hospital in Rome, demonstrated the emergence of carbapenem resistant strains carrying the *bla_{OXA-58}* gene.¹

The aim of this study was to investigate *A. baumannii* isolates collected during the period 2004–2005 in the intensive care unit (ICU) and other wards of the Policlinico Umberto I (hospital A) in Rome. For comparison purpose, isolates of *A. baumannii* from a distantly located hospital of the town, the Madre G. Vannini (hospital B), were also investigated. All strains were genotyped by random amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), detection of the *bla_{OXA-58}* gene and plasmid analysis. Since studies of antibiotic resistance mechanisms in *Acinetobacter* spp. have demonstrated frequent occurrence of integrons in multidrug resistant isolates of this species,^{11,13,18,31} integrons were also searched and characterized in this collection of strains.

MATERIALS AND METHODS

A. baumannii isolates

Fifty-four consecutive *A. baumannii* were isolated from May to July 2004 from 15 patients recovered at the ICU of hos-

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pital A. Fourteen patients had a diagnosis of ventilator-associated pneumonia, based on clinical (pulmonary index score > 5) and microbiologic criteria (positive quantitative cultures of airway specimens with a cutoff of $\geq 10^5$ colony forming units) and one patient was colonized.¹² Fifteen nonrepetitive isolates (1 per patient) were selected for this study, 13 from tracheal or bronchial aspirates and 2 from venous catheters (Table 1). Only two *A. baumannii* isolates were obtained in 2004 from patients hospitalized in different wards of the same hospital (Table 1). In the same year, 19 *A. baumannii* were isolated at the hospital B from 15 patients affected by bloodstream infections, ventilator associated pneumonia or simply colonized. One strain for each patient was included in this study (Table 1).

In 2005, 19 strains were sporadically isolated from different patients at the ICU or in other wards of hospitals A, 11 were also included in this study (those previously characterized¹ were not included) together with two strains isolated in 2005 at hospital B (Table 1).

A. baumannii were isolated from clinical specimens by using standard methods and identified by using the Vitek 2 system with ID-GN cards according to the manufacturer's instructions (bioMérieux, Mercy l'Etoile, France). Antimicrobial susceptibility tests and minimal inhibitory concentrations (MIC) were performed with the Vitek 2 system-AST-GN9 cards (bioMérieux) according to the manufacturer's instructions. Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines.⁴ The following antimicrobial agents at the indicated concentrations were tested: amikacin at 8, 16, and 64 $\mu\text{g/ml}$; ampicillin at 4, 8 and 32 $\mu\text{g/ml}$; ampicillin/sulbactam at 4/2, 16/8, and 32/16 $\mu\text{g/ml}$; aztreonam at 2, 8 and 32 $\mu\text{g/ml}$; cefazolin at 4, 16, and 64 $\mu\text{g/ml}$; cefepime at 2, 8, 16, and 32 $\mu\text{g/ml}$; cefotetan at 2, 8, and 32 $\mu\text{g/ml}$; ceftazidime at 1, 2, 8, and 32 $\mu\text{g/ml}$; ceftriaxone at 1, 2, 8, and 32 $\mu\text{g/ml}$; cefuroxime at 2, 8 and 32 $\mu\text{g/ml}$; ciprofloxacin at 0.5, 2, and 4 $\mu\text{g/ml}$; gentamicin at 4, 16, and 32 $\mu\text{g/ml}$; imipenem at 2, 4, and 16 $\mu\text{g/ml}$; levofloxacin at 0.5, 4, and 8 $\mu\text{g/ml}$; meropenem at 0.5, 4 and 16 $\mu\text{g/ml}$; nitrofurantoin at 16, 32 and 64 $\mu\text{g/ml}$; piperacillin at 4, 16 and 64 $\mu\text{g/ml}$; piperacillin/tazobactam at 4/4, 16/4, and 128/4 $\mu\text{g/ml}$; tobramycin at 8, 16, and 64 $\mu\text{g/ml}$ and trimethoprim/sulfamethoxazole at 0.5/9.5, 2/38 and 16/304 $\mu\text{g/ml}$.

The presence of metallo- β -lactamases was excluded by using the E-test metallo- β -lactamase (MBL) strips³⁵ (AB BIODISK, Solna, Sweden).

RAPD

Genomic DNA preparation of *A. baumannii* isolates was performed by using the Wizard Genomic DNA purification kit (Promega Corporation Madison, WI) according to the manufacturer's instructions. All strains were first genotyped by RAPD, using primer and conditions previously described,³ by the RAPD primer 5'-GCTTGTGAAC-3'.

PFGE

DNA preparation was performed as previously described.³⁴ DNA restriction was done with 50U *Apa*I enzyme (New England Biolabs, Ipswich, MA) and resolved on 1% agarose gel (Bio-Rad Pulsed Field Certified) on a CHEF-DRII system (Bio-Rad Laboratories, Hercules, CA) over 20 hr at 14°C with 5 to

13 sec of linear ramping at 6 V/cm.³⁴ Lambda ladder (New England Biolabs) was used as molecular reference marker. Image of ethidium bromide-stained gel, stored as TIFF file, was analyzed by using the computer software BioNumerics (Applied Maths, Kortrijk, Belgium) using Dice coefficient and unweighted pair group method of averages (UPGMA) with a 1% tolerance limit and 1.0% optimization.

Integron detection and characterization

Class 1 integron detection was performed by polymerase chain reaction (PCR) amplification with the 5'-CS and 3'-CS primers as previously described¹⁹ using 200 ng of genomic DNA extracted by the Wizard Genomic DNA purification kit (Promega Corporation Madison) according to manufacturer's instructions. The 5'-CS-3'-CS PCR products were cloned into the pGEM T-Easy Vector (Promega Corporation Madison) and introduced by transformation in *Escherichia coli* cells INV α (Invitrogen). The sequence of the inserts was fully determined by dye terminator cycle sequencing with the ABI Prism model 377 sequencer (Applied Biosystems, Foster City, CA). Specific primer pairs for the *aacA4* (5'-CGTACGTTGCTCTTGAAG-3'), *bla_{OXA-20}* (5'-CTTTAGGATGGAAATTTGTTC-3') and *orfX* (5'-CCGGCTTAACCTCAGGCGTTG-3') and *aadA1* (5'-TCGATGACGCCAACTAC-3') integron-borne gene cassettes were then used to confirm the gene cassette arrays identified by DNA sequencing of the 5'-CS-3'-CS amplicons from prototypic strains, in the other strains of the collection.

Carbapenem resistance characterization

PCR for detection of the *bla_{IMP}* and *bla_{VIM}* genes was carried out with primer pairs previously described.⁸ The presence of the *bla_{OXA-58}* was investigated by PCR assays on genomic DNAs using previously described primer pairs.^{14,15} The *ISAb_{a3}* element was identified by PCR with the preOXA-58prom+ as forward primer¹⁴ and the *ISAb_{a3}* reverse primer (5'-AGGC-AGGTTGGACATTGATT-3', EMBL AY570763 DNA sequence).

Plasmid analysis

Plasmid DNA was purified by the QIAprep Spin Miniprep Kit (Qiagen). Southern blot hybridization was carried out on the *Eco*RI-restricted plasmids, using a specific *bla_{OXA-58}* probe, obtained amplifying the internal part of the gene, as previously described,¹⁴ and labeling the probe by the PCR DIG Labelling mix procedure (Roche Diagnostics, Monza, Italy).

RESULTS

Genotyping of *A. baumannii* clinical isolates

Forty-five *A. baumannii* strains isolated in two different hospitals in Rome in 2004 and 2005 were initially genotyped by RAPD. Isolates, which differed by two or more prominent bands, were considered sufficiently divergent to warrant separate strain designations.²⁹ Four distinct RAPD patterns were identified (Table 1, Fig. 1). The RAPD-1 type was the most prevalent, being detected in 35 of 45 isolates, namely in all the carbapenem resistant strains, isolated from both hospitals dur-

TABLE 1. CHARACTERISTICS OF *ACINETOBACTER BAUMANNII* ANALYZED IN THIS STUDY

<i>Hospital (ward)</i>	<i>Year</i>	<i>RAPD type</i>	<i>PFGE type</i>	<i>Integron gene cassettes</i>	<i>Amikacin</i>	<i>Gentamicin</i>	<i>Imipenem</i>	<i>bla_{OXA-58}</i>	<i>No. of strains</i>
Hospital A (ICU) ^a	2004	1	1	<i>aacA4</i> , <i>orfO</i> , <i>bla_{OXA-20}</i>	resistant	susceptible	resistant	pos	15
Hospital A (ICU)	2005	1	1	<i>aacA4</i> , <i>orfO</i> , <i>bla_{OXA-20}</i>	resistant	susceptible	resistant	pos	8
Hospital A (OW)	2005	1	1	<i>aacA4</i> , <i>orfO</i> , <i>bla_{OXA-20}</i>	resistant	susceptible	resistant	pos	2
Hospital A (OW)	2004	1	5	<i>aacA4</i> , <i>orfO</i> , <i>bla_{OXA-20}</i>	resistant	susceptible	resistant	pos	1
Hospital A (OW)	2004	1	6	<i>aacA4</i> , <i>orfO</i> , <i>bla_{OXA-20}</i>	resistant	susceptible	resistant	pos	1
Hospital B (ICU)	2004	1	6	<i>aacA4</i> , <i>orfO</i> , <i>bla_{OXA-20}</i>	resistant	susceptible	resistant	pos	6
Hospital B (ICU)	2005	1	6	<i>aacA4</i> , <i>orfO</i> , <i>bla_{OXA-20}</i>	resistant	susceptible	resistant	pos	2
Hospital B (ICU)	2004	2	2	<i>aacC1</i> , <i>orfX</i> , <i>X'</i> , <i>X''</i> , <i>aadA1</i>	susceptible	resistant	susceptible	neg	8
Hospital B (ICU)	2004	3	3	<i>aacC1</i> , <i>orfX</i> , <i>X'</i> , <i>X''</i> , <i>aadA1</i>	susceptible	resistant	susceptible	neg	1
Hospital A (OW)	2005	4	4	—	susceptible	resistant	susceptible	neg	1

^aOutbreak strains.

ICU, intensive care unit; OW, other wards; RAPD, random amplified polymorphism DNA; PFGE, pulsed field gel electrophoresis.

FIG. 1. Random amplified polymorphism DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) profiles obtained for prototypic *Acinetobacter baumannii* isolates. Dendrogram of the PFGE macro restriction patterns obtained after digestion of genomic DNA with the *ApaI* restriction enzyme. PFGE profiles were analyzed with the Bionumerics computer software and designated as types 1, 2, 3, 4, 5, 6. The scale indicates percent of similarity. RAPD profiles designated as types 1, 2, 3, and 4 from representative *A. baumannii* strains. M lane contains the 1 kb molecular mass marker (Invitrogen, Milan, Italy).

ing the 2-year period. The RAPD-1 type was highly recurrent in hospital A, being detected in all isolates except one showing the RAPD-4 type, isolated in 2005 and showing susceptibility to carbapenems (Table 1). Eight strains isolated in 2004 in hospital B showed a different RAPD type, designated as RAPD-2, and one strain showed a profile classified as RAPD-3 (Table 1).

To increase the discriminatory power of the RAPD genotyping, all strains were then analyzed by PFGE, after DNA digestion with the *ApaI* enzyme. Using a $\geq 88\%$ similarity cut-off point, PFGE identified 6 different *ApaI* digest profiles among the *A. baumannii* strains. Three different PFGE types, designated as PFGE-1, PFGE-5, and PFGE-6 were recognized within the group of strains classified as RAPD-1. Three PFGE types, designated as PFGE-2, PFGE-3, and PFGE-4 were assigned to the strains showing the RAPD-2, RAPD-3, and RAPD-4 types, respectively (Table 1, Fig. 1).

Identical RAPD and PFGE patterns (RAPD-1, PFGE-1) were obtained among the 15 strains isolated in summer 2004 at hospital A, defining the genotype of an epidemic clone causing an outbreak at the ICU in that period.

Antimicrobial resistance profiles

The antimicrobial resistance and MICs were determined for all the strains (Table 1). Interestingly, the *A. baumannii* strains of the RAPD-1 type all exhibited a multidrug resistant pheno-

type characterized by resistance to imipenem, aztreonam, piperacillin-tazobactam, broad-spectrum cepheims, fluoroquinolones, and amikacin, but they were all susceptible to gentamicin (MIC, 4 mg/L). The *A. baumannii* RAPD-2 and -3 were all susceptible to imipenem and amikacin but were resistant to broad-spectrum cepheims, aztreonam, and gentamicin (MIC, > 16 mg/L). The RAPD-4 isolate showed a susceptible phenotype including susceptibility to imipenem, gentamicin and amikacin (Table 1).

Characterization of class 1 integrons in the *A. baumannii* isolates

All strains were analyzed for integron content by PCR amplification with the primers 5'-CS and 3'-CS,¹⁹ revealing that the two prevalent clones of RAPD-1 and -2 types carried two different integrons of 2.2 kb and 2.5 kb variable regions, respectively. Nucleotide analysis of the RAPD-1-type-associated 2.2 kb integron showed that this integron contains the *aacA4-orfO-bla_{OXA-20}* gene cassettes, conferring resistance to amikacin and to several β -lactams. Nucleotide sequences of the RAPD-2 and -3-type-associated 2.5 kb integron identified the *aacC1-orfX-X'-X''-aadA1* gene cassettes, conferring resistance to gentamicin and streptomycin/spectinomycin. Integron-borne gene cassettes perfectly correlate with the aminoglycoside resistance patterns characterizing the RAPD-1 and RAPD-2 types, showing amikacin or gentamicin resistance, respectively.

Plasmid-mediated carbapenem-hydrolyzing oxacillinase OXA-58

All strains were tested for the *bla*_{OXA-58} gene and all the carbapenem-resistant strains, from both hospitals A and B, isolated in 2004 and in 2005 were positive for this oxacillinase (Table 1). Since the *bla*_{OXA-58} gene has been demonstrated to be associated with the insertion sequence IS_{Aba2},¹⁴ the presence of this mobile element was also investigated and positively identified in the 10 prototypic carbapenem resistant strains (the susceptible strain was negative to this amplification, data not shown). The *bla*_{OXA-58} amplicons obtained from 10 different strains were fully sequenced, demonstrating 100% identity of the amplified gene with the *bla*_{OXA-58} sequence (EMBL accession no. AY570763).²⁵

A. baumannii strains were analyzed by plasmid profiling: slightly different restriction patterns were obtained for the carbapenem resistant epidemic clone of the hospital A with respect to the strains isolated at the hospital B in the same year (Fig. 2). In order to localize the *bla*_{OXA-58} gene on these plasmids Southern blot hybridization experiments were performed with a *bla*_{OXA-58} gene specific probe, on *Eco*RI restricted plasmids purified from 5 strains representative of the epidemic clone (RAPD-1, PFGE-1), 3 carbapenem resistant sporadic strains isolated at the hospital B (RAPD-1, PFGE-6) and from the susceptible RAPD-3 strain, used as negative control. The *bla*_{OXA-58} gene was localized on an identical *Eco*RI fragment of approximately 11.5 kb in the five strains representative of

the epidemic clone, while the unrelated strains showed different *Eco*RI fragments (ranging from 14 kb to 9 kb), indicating a certain variability in the *bla*_{OXA-58} gene localization among the unrelated isolates and the epidemic clone (Fig. 2).

DISCUSSION

Infections with *A. baumannii* are of great concern for hospitalized patients, showing the highest individual rate of incidence, particularly, in the intensive care units. Multidrug-resistant clones of *A. baumannii* are spreading throughout many different geographic areas, and susceptible strains are acquiring resistance genes.^{6,10,30,33,37} Treatment options for multidrug-resistant *A. baumannii* infections are limited in most cases to carbapenems, but the emergence of imipenem resistance mandates the adoption of alternative therapeutic options, among which the use of old drugs such as sulbactam and colistin is to be considered.^{17,21,23}

We characterized 45 *A. baumannii* strains circulating in two different hospitals in Rome, demonstrating the presence of a clonal strain associated with an outbreak occurred at the intensive care unit of the Policlinico Umberto I of Rome. Retrospectively, the outbreak involved 14 cases of infection by multidrug-resistant *A. baumannii* over a 3-month period. Molecular genotyping by PFGE analysis was sensitive in detecting slightly diverging subtypes within the same RAPD-type, but a unique

FIG. 2. Plasmid localization of the *bla*_{OXA-58} gene. Agarose (1.0%) gel electrophoresis in 1× TAE buffer of *Eco*RI-digested plasmids from 8 *Acinetobacter baumannii* isolates, stained with ethidium bromide and visualized under UV light, and Southern blot hybridization with the *bla*_{OXA-58} probe. M1 is 1 kb Plus Ladder (Invitrogen, Milan, Italy). The asterisk indicates the carbapenem susceptible strain used as negative control for the Southern blot hybridization with the *bla*_{OXA-58} probe.

RAPD-1–PFGE-1 profile was observed for the epidemic clone. Strains with identical genotype were also detected at the intensive care unit and in other wards of the Policlinico Umberto I in 2005, suggesting the recurrence of this epidemic clone and its spread within this hospital.

A second recurrent genotype (RAPD-2–PFGE-2) was observed in M.G. Vannini of Rome. PCR identification and characterization of integrons provided good markers for the characterization of the aminoglycoside resistance in the RAPD-1 and RAPD-2 isolates. Two specific integron-borne gene cassette arrays conferring amikacin or gentamicin resistance, clearly correlated with the two prevalent RAPD-1 and -2 types, respectively. The gene cassette arrays of these integrons were previously described in several clinical isolates of *A. baumannii* from different countries, including Italy.^{11,31,37} In particular, the integron carrying the *aacC1-orfX-X'-X''-aadA1* gene cassettes has been recently demonstrated to be integrated in a large resistance island in the chromosome of the AYE epidemic strain of *A. baumannii* from France,⁹ suggesting a global diffusion of this resistance determinant. Noteworthy, only one strain lacks integrons among the isolates of our collection and it was isolated from a patient with cystic fibrosis, presumably representing a community-acquired infection.

Integron-borne gene cassettes did not confer the carbapenem resistance that was identified on plasmids carrying the *bla*_{OXA-58} gene. This gene was detected in all the carbapenem-resistant isolates, independently on hospital of origin or period of isolation, demonstrating that this gene is widely diffused. This study emphasizes the importance of the *bla*_{OXA-58} carbapenemase gene in conferring carbapenem resistance among *A. baumannii* isolates in Italy and worldwide, as demonstrated by the increasing number of reports in the recent literature, describing OXA-58-producing *A. baumannii* isolates in several countries of three continents.^{2,5,20,27}

In our study, the analysis of the *bla*_{OXA-58} plasmid contributed to the description of the outbreak clone, since a conserved *bla*_{OXA-58} plasmid characterized the epidemic strain with respect to other plasmid variants identified in unrelated isolates, suggesting that plasmid profiles may help in epidemiological investigations.²²

Prospective surveillance programs by characterization of antibiotic resistance, genotyping and plasmid analysis will be useful to follow the recurrence of the described *A. baumannii* clone as well as to rapidly detect genotype and resistance pattern changes in new emerging clones in our country.

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