ORIGINAL ARTICLE 10.1111/j.1469-0691.2009.02668.x

Epidemic multidrug-resistant *Acinetobacter baumannii* related to European clonal types I and II in Rome (Italy)

S. D'Arezzo[1], A. Capone[1], N. Petrosillo[1] and P. Visca[1,2], on behalf of GRAB[3]

1) *National Institute for Infectious Diseases "Lazzaro Spallanzani", I.R.C.C.S., Rome,* 2) *Department of Biology, University "Roma Tre", Rome, Italy and* 3) *GRAB (Gruppo Romano Acinetobacter baumannii) members are: M. Ballardini, S. Bartolini, E. Bordi, A. Di Stefano, M. Galiè, R. Minniti, M. Meledandri, L. Pacciani, G. Parisi, G. Prignano, C. Santini, M. Valmarin, M. Venditti, S. Ziantoni*

Abstract

The molecular epidemiology and the genetic basis of antibiotic resistance in 88 multidrug-resistant (MDR) *Acinetobacter baumannii* strains isolated during 18 months from infected patients in seven intensive care units (ICUs) in Rome were investigated. Random amplified polymorphic DNA and macrorestriction analysis identified two predominant clonal types, genetically related to the European epidemic clones I (type 2) and II (type 1), accounting for 98.9% of *A. baumannii* ICU isolates. Type 1 was isolated from all ICUs under survey. Class 1 integrons of 2.2 and 2.5 kb were detected in type 1 and type 2 isolates, respectively. The integron structures were similar to those previously determined for epidemic *A. baumannii* strains from various European countries, and suggestive of integron rearrangement/exchange among isolates related to the European epidemic clones I and II. Carbapenem resistance was associated with the presence of the $bla_{OXA\text{-}58}$ gene in type 1 isolates. The results indicate that the *A. baumannii* type 1 clone has a high potential of spreading among hospitals.

Keywords: *Acinetobacter baumannii,* epidemiology, integrons, multidrug resistance, nosocomial infection, typing
Original Submission: 29 March 2008; **Revised Submission:** 10 June 2008; **Accepted:** 19 June 2008
Editor: R. Cantón
Clin Microbiol Infect 2009; **15:** 347–357

Corresponding author and reprint requests: P. Visca, Dipartimento di Biologia, Università di Roma Tre, Viale G. Marconi 446, 00146 Roma, Italia
E-mail: visca@uniroma3.it

Introduction

Acinetobacter baumannii infection is a leading cause of morbidity and mortality in the hospital setting, especially among critically ill patients in intensive care units (ICUs). The epidemiology of *A. baumannii* infection in ICUs is complex due to the coexistence of epidemic cases with unrelated sporadic cases caused by different strains [1,2]. Outbreaks of *A. baumannii* infection in ICUs have often been attributed to transmisson via ventilatory equipment and to hand transmission by health care personnel [1,2]. The epidemic potential and the clinical severity of *A. baumannii* infections are related to resistance of the isolates to antimicrobial agents, including broad-spectrum β-lactams, aminoglycosides, fluoroquinolones and carbapenems [3].

A few lineages of *A. baumannii* have caused multiple hospital outbreaks in different countries, and have therefore acquired epidemic status. By convention, these lineages are termed clones and their relatedness is assessed on a genotypic basis [4]. Two pan-European epidemic clones of *A. baumannii*, referred to as European clones I and II [5,6], became widespread in north-western Europe in the years 1982–1990 [5] and prevailed in the Czech Republic from 1991 to 2001 [6,7]. With time, clone I was isolated in Spain, Poland, the UK and Italy, while clone II was obsrved in Spain, Portugal, France, Greece, the UK and Turkey [8–10]. A third pan-European *A. baumannii* clone, clone III, probably persisting in European hospitals since the 1990s, has recently been described in France, the Netherlands, Italy, and Spain [10,11].

Multidrug resistance in *A. baumannii* is linked to the presence of resistance islands, mobile genetic elements and integrons capable of capturing antibiotic resistance genes by site-specific recombination [2,12–14]. In fact, class 1 integrons with different variable regions have been identified in pan-European clones I, II and III [11].

Few reports have been published concerning the epidemiology of nosocomial *A. baumannii* infection in Italy [15–20], and a systematic survey of the hospital epidemiology and drug resistance of *A. baumannii* is not yet available on a national scale. Recently, the circulation of different *A. baumannii* clones, including a carbapenem-resistant one,

has been documented in three apparently unrelated ICU outbreaks which occurred during 2004–2005 in Rome [18–20]. In this scenario, a network of laboratories and clinicians (Gruppo Romano *Acinetobacter baumannii* (GRAB)) was created with the aim of tracing the hospital epidemiology of *A. baumannii* infection in the Rome urban area (including seven hospitals with a total of 5392 beds, 187 of which were in ICUs), and providing collaborating centres with outbreak alerts, antibiotic-resistance surveillance data and typing facilities.

This study provides an in-depth characterization of 88 multidrug-resistant (MDR) *A. baumannii* strains responsible for seven ICU outbreaks in Rome, with the aim of defining their epidemiological traits and the genetic basis of antibiotic resistance.

Materials and Methods

Bacterial isolates

A total of 90 MDR isolates, provisionally identified as *A. baumannii*, was collected from outbreaks or sporadic cases of infection that occurred during the 18-month investigation period (from 7 January 2004 to 27 June 2005) in seven ICUs, designated A, B, C, D, E, F, G, of large regional hospitals in the Rome urban area, and submitted to the coordinating laboratory at the National Institute for Infectious Diseases 'Lazzaro Spallanzani' (Rome) for further characterization (Table 1). The index strains (study codes 97 and 115) from previously published outbreaks were provided by hospitals F and G [19,20], in addition to two MDR isolates recovered from sporadic cases preceding the outbreak in hospital F. During the outbreaks, local control of the epidemics required urgent measures, including environmental sampling, aggressive environmental disinfections, and, at least in two hospitals, temporary closure of the ICU admissions.

Bacteria were routinely identified to the species level by the participating centres using the Phoenix (Becton Dickinson, Sparks, MD, USA) and Vitek 2 (BioMérieux, Marcy-l'Etoile, France) commercial systems. Isolates were from respiratory secretions (n = 49), central venous catheters (n = 11), urine (n = 8), wound swabs (n = 6), blood (n = 6), and cerebrospinal fluid (n = 1), and were all associated with a clinical infection. Only the primary isolate of each infected patient was included in the collection. Nine environmental isolates were recovered from medical devices or the ICU environment of hospitals B and C (Table 1). The two reference strains for epidemic European clones I (RUH875) and II (RUH134) [5,6] were included in the study for comparison.

Antimicrobial susceptibility testing

Antimicrobial agents tested were ampicillin–sulbactam (SAM), piperacillin (PIP), piperacillin–tazobactam (TZP), cefepime

TABLE 1. Characteristics of *Acinetobacter* strains

Study code (hospital)	Source (isolation date; month/day/year)	Study code (hospital)	Source (isolation date; month/day/year)	Study code (hospital)	Source (isolation date; month/day/year)
1 (A)	Respiratory secretions (06/01/04)	35 (C)	Urine (04/05/04)	74 (C)	Respiratory secretions (05/28/05)
4 (A)	Central venous catheter (08/25/04)	37 (C)	Central venous catheter (04/07/05)	75 (C)	Wound swab (05/17/05)
5 (A)	Respiratory secretions (06/15/04)	38 (C)	Respiratory secretions (04/21/05)	76 (C)	Respiratory secretions (05/30/05)
6 (A)	Central venous catheter (08/02/04)	40 (C)	Respiratory secretions (03/29/05)	77 (C)	Respiratory secretions (06/09/05)
7 (A)	Central venous catheter (07/29/04)	41 (C)	Respiratory secretions (02/14/05)	78 (C)	Urine (06/11/05)
8 (B)	Respiratory secretions (05/06/04)	42 (C)	Urine (04/18/05)	79 (C)	Respiratory secretions (05/03/05)
9 (B)	Blood culture (05/08/04)	43 (C)	Respiratory secretions (07/12/04)	105 (C)	Cerebrospinal fluid (06/27/05)
10 (B)	Respiratory secretions (05/09/04)	44 (C)	Respiratory secretions (07/26/04)	80 (D)	Wound swab (01/24/05)
11 (B)	Respiratory secretions (05/15/04)	46 (C)	Respiratory secretions (03/21/05)	81 (D)	Respiratory secretions (02/15/04)
12 (B)	Respiratory secretions (05/18/04)	48 (C)	Respiratory secretions(03/29/05)	82 (D)	Wound swab (04/12/04)
13 (B)	Respiratory secretions (05/28/04)	50 (C)	Respiratory secretions (01/07/04)	83 (D)	Central venous catheter (10/14/04)
14 (B)	Respiratory secretions (06/14/04)	51 (C)	Respiratory secretions (11/16/04)	84 (D)	Urine (03/19/04)
16 (B)	Respiratory secretions (06/21/04)	52 (C)	Respiratory secretions (07/05/04)	85 (D)	Respiratory secretions (03/04/04)
17 (B)	Respiratory secretions (07/06/04)	53 (C)	Urine (03/09/04)	86 (D)	Urine (04/11/04)
19 (B)	Respiratory secretions (09/15/04)	54 (C)	Respiratory secretions (01/19/04)	87 (D)	Central venous catheter (07/06/04)
20 (B)	Respiratory secretions (09/28/04)	55 (C)	Urine (01/13/04)	88 (D)	Respiratory secretions (03/02/04)
21 (B)	Central venous catheter (09/28/04)	57 (C)	Respiratory secretions (02/25/04)	89 (D)	Respiratory secretions (02/11/05)
22 (B)	Respiratory secretions (08/18/04)	58 (C)	Blood culture (06/16/04)	90 (D)	Wound swab (03/03/05)
23 (B)	Respiratory secretions (10/11/04)	60 (C)	Respiratory secretions (07/26/04)	91 (D)	Central venous catheter (03/10/05)
24 (B)	Blood culture (10/16/04)	61 (C)	Respiratory secretions (03/15/04)	92 (D)	Blood culture (05/10/05)
25 (B)	Urine (11/23/04)	62 (C)	Wound swab (03/19/04)	93 (D)	Central venous catheter (10/04/04)
26 (B)	Environmental, laryngoscope (05/28/04)	63 (C)	Respiratory secretions (06/21/04)	94 (D)	Blood culture (10/01/04)
27 (B)	Environmental, ventilator (06/14/04)	64 (C)	Respiratory secretions (03/24/05)	95 (D)	Respiratory secretions (10/26/04)
28 (B)	Environmental, laryngoscope (06/21/04)	66 (C)	Respiratory secretions (10/19/04)	96 (D)	Central venous catheter (08/05/04)
29 (B)	Central venous catheter (07/19/04)	67 (C)	Environmental, cabinet (06/16/04)	73 (E)	Respiratory secretions (05/28/05)
30 (B)	Respiratory secretions (07/15/04)	68 (C)	Environmental, trolley (06/21/04)	97 (F)[a]	Respiratory secretions (06/25/05)
31 (B)	Respiratory secretions (07/19/04)	69 (C)	Environmental, trolley (07/05/04)	98 (F)	Respiratory secretions (06/07/05)
32 (B)	Respiratory secretions (07/13/04)	70 (C)	Environmental,ventilator (07/12/04)	99 (F)	Respiratory secretions (11/19/04)
33 (C)	Wound swab (03/10/04)	71 (C)	Environmental, desk surface (07/26/04)	100 (F)	Respiratory secretions (03/01/05)
34 (C)	Respiratory secretions (09/30/04)	72 (C)	Environmental, desk surface (07/26/04)	115 (G)[a]	Blood culture (06/10/05)

[a]*A. baumannii* index strains [19,20]; study code 115 is equivalent to strain ACICU [13].

(FEP), ceftazidime (CAZ), aztreonam (ATM), imipenem (IPM), meropenem (MEM), amikacin (AMK), gentamicin (GEN), ciprofloxacin (CIP), levofloxacin (LVX), trimethoprim-sulfamethoxazole (SXT), colistin (CS), and tigecycline (TIG). For all antimicromials, except CS and TIG, susceptibility testing was performed with the Vitek 2 system. MIC results were interpreted according to the CLSI breakpoint criteria [21]. TIG and CS susceptibility was determined by the broth microdilution method [21]. The MICs of TIG were determined in 96-well microtiter plates (Costar, Cambridge, MA, USA) containing freshly prepared Mueller–Hinton broth (Oxoid, Milan, Italy). The inoculum was adjusted to *c.* 5×10^5 CFU/mL, and plates were visually read after incubation for 24-h incubation at 37°C. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as internal quality control strains. The US FDA breakpoints approved for Enterobacteriaceae were applied to define TIG susceptibility (susceptibility, ≤2 mg/L; resistance, ≥8 mg/L). The criteria proposed by Gales *et al.* [22] were used for interpretation of CS susceptibility. The MDR phenotype was defined as diminished susceptibility to ≥2 of the following drug classes: antipseudomonal cephalosporins, antipseudomonal carbapenems, β-lactam–β-lactamase inhibitor combinations, antipseudomonal fluoroquinolones and aminoglycosides [23].

Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA was carried out with restriction enzymes AluI, CfoI, MboI, MspI, and RsaI (Roche, Monza, Italy), as previously described [24]. Ribosomal (r)DNA restriction patterns were interpreted according to Dijkshoorn *et al.* [24] and Vaneechoutte *et al.* [25].

Epidemiological typing

Random Amplified Polymorphic DNA (RAPD) analysis was performed for all isolates with M13, ERIC-2, DAF4 and decanucleotide primers (Table 2) as previously described [26,27].

Macrorestriction analysis of *Apa*I-digested genomes was performed for a selected group of *A. baumannii* isolates, representative of the different RAPD types and hospitals. Pulsed-field gel electrophoresis (PFGE) was carried out as previously described [28], using a CHEF mapper (Bio-Rad, Segrate, Milan, Italy). PFGE profiles were interpreted according to published criteria [29], with a difference of six bands or less used to define epidemiological relatedness.

Electropherograms were analysed either visually or using the Bionumerics software (Applied Maths, Sint-Martems-Latem, Belgium). The percentage of pattern similarity between pairs of isolates was calculated as (number of shared fragments × 2 × 100)/total number of fragments in the two samples [30]. The BioNumerics analysis was performed using the Dice coefficient and the unweighted pair group method of averages (UPGMA) with a 1% tolerance limit and 1% optimisation. Isolates that clustered with ≥80% or ≥65% similarity were considered to belong to the same RAPD or PFGE type, respectively. Within the same RAPD type, isolates differing by <2 bands were considered subtypes. Multiplex PCRs for the definition of *A. baumannii*

TABLE 2. List of primers used in the study

Primer	Nucleotide sequence (5′–3′)	Amplicon size (bp)	References
intI1-FW	CAGTGGACATAAGCCTGTTC	160	[14]
intI1-RV	CCCGAGGCATAGACTGTA		
intI2-FW	TTGCGAGTATCCATAACCTG	288	[14]
intI2-RV	TTACCTGCACTGGATTAAGC		
intI3-FW	GCCTCCGGCAGCGACTTTCAG	979	[32]
intI3-RV	ACGGATCTGCCAAACCTGACT		
5′CS	GGCATCCAAGCAGCAAG	V	[14]
3′CS	AAGCAGACTTGACCTGA		
16S-FW	TGGCTCAGATTGAACGCTGGC	≅ 1500	[25]
16S-RV	TACCTTGTTACGACTTCACCCCA		
bla_{IMP}-FW	ATGAGCAAGTTATCTGTATTCT	741	[16]
bla_{IMP}-RV	TTAGTTGCTTGGTTTTGATGG		
bla_{OXA-23}-FW	GATCGGATTGGAGAACCAGA	501	[35]
bla_{OXA-23}-RV	ATTTCTGACCGCATTTCCAT		
bla_{OXA-24}-FW	GGTTAGTTGGCCCCCTTAAA	246	[35]
bla_{OXA-24}-RV	AGTTGAGCGAAAAGGGGATT		
bla_{OXA-51}-FW	TAATGCTTTGATCGGCCTTG	353	[35]
bla_{OXA-51}-RV	TGGATTGCACTTCATCTTGG		
bla_{OXA-58}-FW	CGATCAGAATGTTCAAGCGC	528	[34]
bla_{OXA-58}-RV	ACGATTCTCCCCTCTGCGC		
ISAba1-FW	CACGAATGCAGAAGTTG	V	[36]
ERIC-2	AAGTAAGTGACTGGGGTGAGCG	M	[26]
M13	GAGGGTGGCGGTTCT	M	[26]
DAF4	CGGCAGCGCC	M	[26]
Decanucleotide	GCTTGTGAAC	M	[27]
M13-FW	GTAAAACGACGGCCAGT	V	[33]
M13-RV	AACAGCTATGACCATG		

M, multiple; V, variable.

sequence groups 1 and 2 were performed using *ompA*, *csuE* and bla_{OXA-51}-like target genes as described [31].

Integron analysis, cloning and sequencing

Integrons were searched for by PCR with primer pairs targeting the *int*I1, *int*I2 or *int*I3 genes (Table 2), as described [14,32]. The internal variable region encompassing the gene cassettes of class 1 integrons was amplified with primers 5′-CS and 3′-CS (Table 2) [14]. For preliminary comparison of integrons, restriction fragment length polymorphysm (RFLP) analysis with *Hin*fI (Roche) was performed for the pool of amplicons generated from each strain with primers 5′-CS and 3′-CS. Then, individual integron bands from prototypic strains were extracted from agarose, purified using the Quiquick gel extraction kit (Qiagen, Milan, Italy), digested with *Hin*fI, and subjected to electrophoresis to define integron-specific RFLPs. Purified integron bands were also ligated into the pDrive plasmid (Qiagen), used to transform *E. coli* DH5α competent cells [33], and sequenced. Nucleotide sequence similarity searches were performed using the BLAST tool in the GenBank database (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi).

Identification of carbapenem-resistance genes

The presence of the bla_{IMP}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51}, and bla_{OXA-58} genes was investigated by PCR as previously reported [16,34,35] (Table 2). The occurrence of the activating IS*Aba1* element upstream of the bla_{OXA-51} gene was determined by PCR with the IS*Aba1*F-OXA-51-likeR primer pair as described [36]. The identity of bla_{OXA-58} and bla_{OXA-24} amplicons was confirmed by direct DNA sequencing.

Results

Identification of *Acinetobacter* genospecies by ARDRA

Eighty-eight of the 90 isolates submitted were definitively identified as *A. baumannii*, showing the typical ARDRA profiles '11123' (72 isolates; 81.8%) and '11121' (16 isolates; 18.2%) with *Cfo*I, *Alu*I, *Mbo*I, *Rsa*I, and *Msp*I, respectively (Fig. S1). Two isolates provisionally identified as *A. baumannii* were assigned to the *Acinetobacter* genospecies 10 (study code 61; ARDRA profile '42123') and 13TU (study code 98; ARDRA profile '21113').

A. baumannii genotyping and correlation with European clones I and II

Five distinct RAPD profiles obtained with M13 and ERIC-2 primers were visually identified, while complex profiles with some unresolved bands were generated by DAF4 (Fig. S2). Unsatisfactory results were obtained using the decanucleotide (data not shown).

The majority of *A. baumannii* strains were grouped in the RAPD-1 type (71 isolates including subtype 1a; 80.7%) and RAPD-2 type (16 isolates including subtype 2a; 18.2%). The two index strains (study codes 97 and 115) showed identical RAPD-1 profiles (Fig. S2). The dendrograms obtained for M13, ERIC-2 and DAF4 RAPD fingerprints highlight the two major *A. baumannii* clusters, corresponding to RAPD-1 and RAPD-2 types (Fig. 1). The two groups are defined by similarity thresholds ≥85%, 90% and 80% for primers M13, ERIC-2 and DAF4, respectively (Fig. 1 and Fig. S2). RAPD-1 and RAPD-2 types were associated with ARDRA profiles '11123' and '11121', respectively. Environmental isolates from hospitals B and C showed the same RAPD-1 type as their clinical counterparts.

The possible correlation between *A. baumannii* RAPD-1 and RAPD-2 types and the European epidemic clones I and II was preliminarily investigated by comparing the RAPD fingerprints of selected strains generated using the M13 and ERIC-2 primers. Significant similarity (100–78%, depending on the primer) was observed between representative RAPD-1 and RAPD-2 type isolates and the European clones II and I, respectively (Fig. 2).

Assuming that *A. baumannii* isolates with identical RAPD profiles from each hospital represent a single strain, a total of 12 *A. baumannii* strains was selected for comparative PFGE analysis with the reference European clones I and II. Two major *Apa*I macrorestriction profiles (pulsotypes 1 and 2) were identified, corresponding to RAPD-1 and RAPD-2 types, respectively (Fig. 2). Interpretation of PFGE patterns confirmed the relationship between *A. baumannii* pulsotypes 1 and 2 and the European clone II and I, respectively (Fig. 2), which formed two clusters defined by the similarity threshold ≥65% (data not shown).

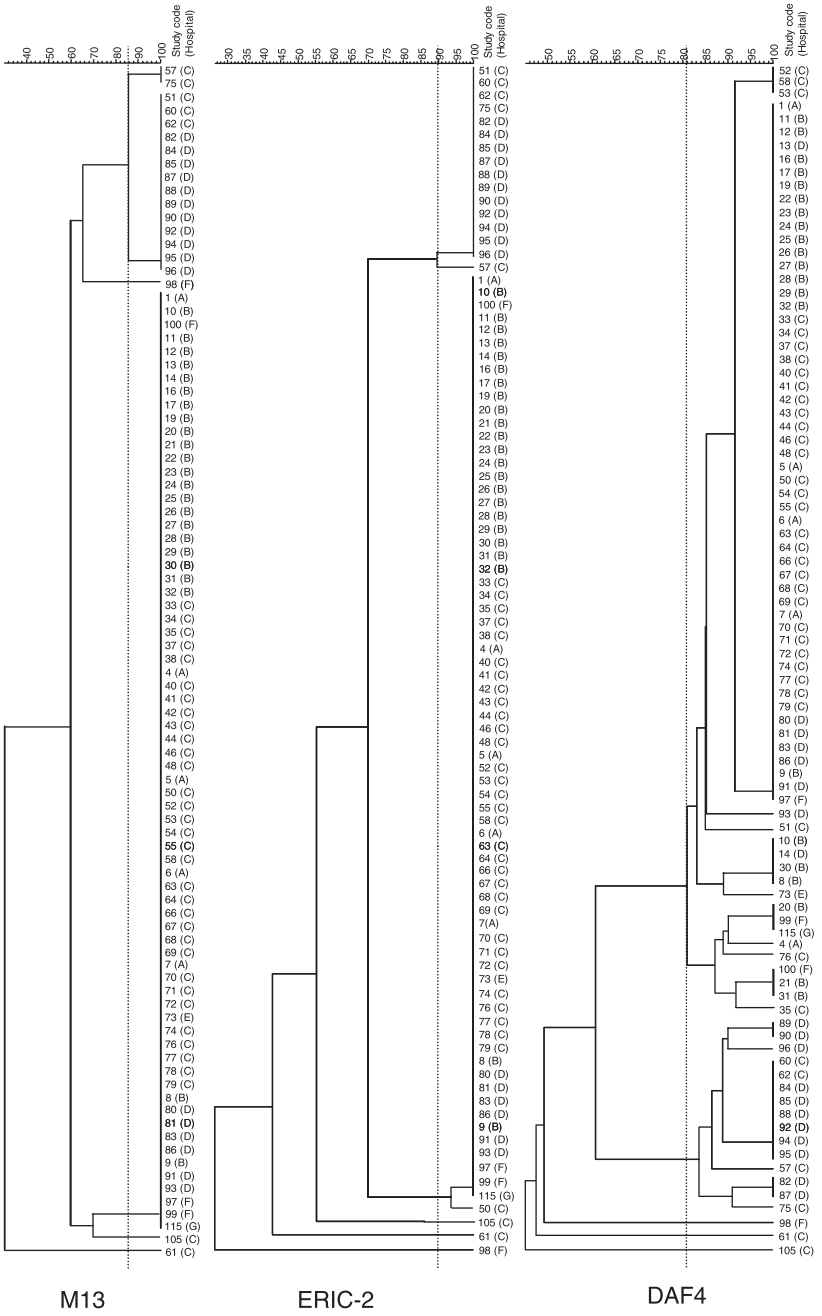
Multiplex PCRs for identification of sequence groups yielded the 111 and 222 allelic profiles (corresponding to sequence types 1 and 2, respectively) for strains belonging to RAPD types 1 and 2, consistent with their genetic relatedness with European clones II and I, respectively (Fig. 2).

Antimicrobial susceptibility

The comparison of antibiotic resistance profiles between *A. baumannii* isolates belonging to RAPD-1 and RAPD-2 types is shown in Fig. 3. *A. baumannii* RAPD-1 and RAPD-2 isolates showed a common MDR profile characterized by resistance to PIP, TZP, CAZ, ATM, CIP, LVX and FEP.

All RAPD-1 type isolates were resistant to carbapenems (IPM and MEM), while RAPD-2 isolates were all susceptible. The MICs of IPM for resistant isolates were in the range of

(a)



(b)

No. of strains (%)	RAPD type	ARDRA pattern (genospecies)
70 (77.8%)	1	11123 (*A. baumannii*)
14 (15.5%)	2	11121 (*A. baumannii*)
1 (1.1%)	1a	11123 (*A. baumannii*)
2 (2.2%)	2a	11121 (*A. baumannii*)
1 (1.1%)	3	42123 (*Acinetobacter* genospecies 10)
1 (1.1%)	4	11123 (*A. baumannii*)
1 (1.1%)	5	21113 (*Acinetobacter* genospecies 13TU)

FIG. 1 RAPD analysis of 90 *Acinetobacter* isolates and correlation with ARDRA types. (a) Clustering relationships inferred from RAPD analysis with M13, ERIC-2, and DAF4 primers. The dendrogram was generated with BioNumerics (Applied Maths) using the unweighted pair-group method with arithmetic averages (UPGMA) and the Dice coefficient. Dotted lines denote the threshold values set to define RAPD types. The strain code, as presented in Table 1, is shown on the right. (b) Association of RAPD types with ARDRA profiles.

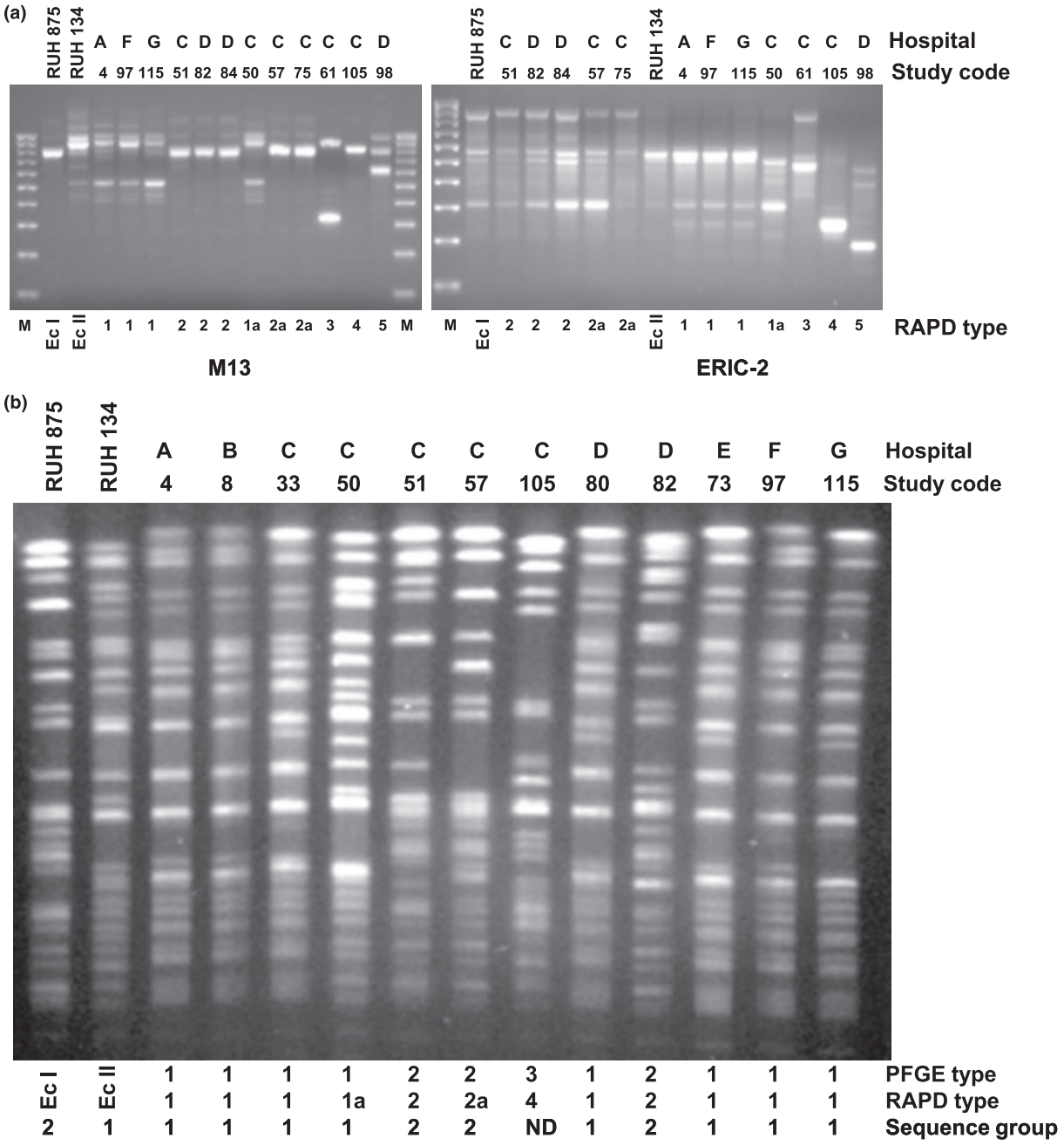


FIG. 2 Comparison of DNA fingerprints among the European epidemic clones I (strain RUH875) and II (strain RUH134) and representative *Acinetobacter baumannii* strains. (a) RAPD profiles obtained with M13 and ERIC-2 primers for strains representative of different hospitals and RAPD types, and reference strains RUH875 and RUH134. The 100 bp DNA size standard (Promega, Milan, Italy) is shown in lanes M. (b) Macrorestriction analysis with enzyme *Apa*I of 12 representative *A. baumannii* isolates. The study code or strain designation and the hospital from which the strain originated are indicated above each lane. RAPD types, pulsotypes, and sequence groups are indicated below each lane. The RAPD-4 type corresponds to pulsotype 3. Ec, European clone; ND, not determined.

16–32 mg/L, except for one RAPD type 1a strain (study code 50) which showed an MIC value of 128 mg/L.

All *A. baumannii* isolates were susceptible to CS, except the only RAPD type 1a strain (study code 50) showing resistance (MIC > 32 mg/L). Interestingly, a significant percentage of type 1 and type 2 *A. baumannii* isolates were susceptible to SAM (48.6% and 71.4% respectively) at MICs ≤ 4–8 mg/L. *A. baumannii* isolates belonging to both types 1 and 2 were

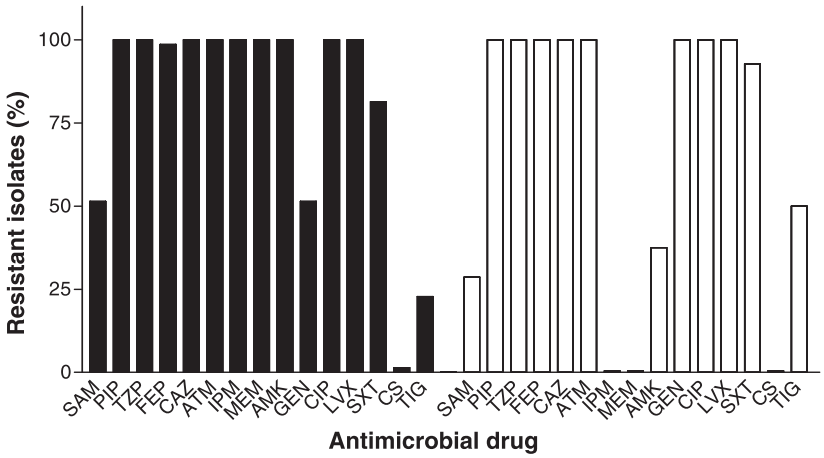


FIG. 3 Antibiotic resistance among 87 *Acinetobacter baumannii* strains belonging to RAPD-1 (black) and RAPD-2 (white) types. Isolates showing an intermediate level of susceptibility were classified as resistant. (a) SAM, ampicillin-sulbactam; PIP, piperacillin; TZP, piperacillin-tazobactam; FEP, cefepime; CAZ, ceftazidime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; LVX, levofloxacin; SXT, trimethoprim-sulfamethoxazole; CS, colistin; TIG, tigecycline.

resistant to TIG at a relatively high rate (27.4% overall, including intermediate and fully resistant isolates; Fig. 3). The MIC_{50} values were 2 and 4 mg/L for type 1 and type 2 isolates, respectively.

Characterization of integrons and antibiotic resistance determinants

The 160 bp DNA fragment internal to the class 1 integrase (*int*I1) gene was amplified from 84 (95.4%) *A. baumannii* isolates, with the exception of RAPD-1a, -2a, and -4 types. All isolates were negative for class 2 and class 3 integrons (data not shown).

Amplification products of *c.* 2.2 and 2.5 kb were obtained with the 5′-CS, 3′-CS primer pair from *A. baumannii* isolates belonging to RAPD-1 and RAPD-2 types, respectively. An additional amplicon of 0.75 kb was common to both types. Products of 0.7 and 3.0 kb were obtained for the European clones I (strain RUH875) and II (strain RUH134), respectively (Fig. S3), corresponding in size to those previously identified by Nemec *et al.* [11]. No amplification products were obtained from *A. baumannii* strains with RAPD types 1a, 2a, and 4 consistent with the absence of the *int*I1 gene in these strains (data not shown).

A preliminary RFLP analysis showed the same integron content for all strains of the same RAPD type, and substantial similarity between the 2.5-kb integron of type 2 strains and the 3.0-kb integron of the European clone II (Fig. S3 and Table S1).

Sequencing of the cloned 2.2-kb variable region revealed the presence of three gene cassettes: the *aacA4* gene encoding an AAC(6')-Ib aminoglycoside acetyltranferase, an open reading frame (ORF) encoding an unknown product, and the bla_{OXA-20} gene encoding a class D β-lactamase (Fig. 4). Sequence analysis of the cloned 2.5 kb variable region revealed the presence of four gene cassettes: the *aacC1* gene encoding an AAC(3)-Ia aminoglycoside acetyltransferase, two ORFs for unknown products, and the *aadA1a* gene encoding

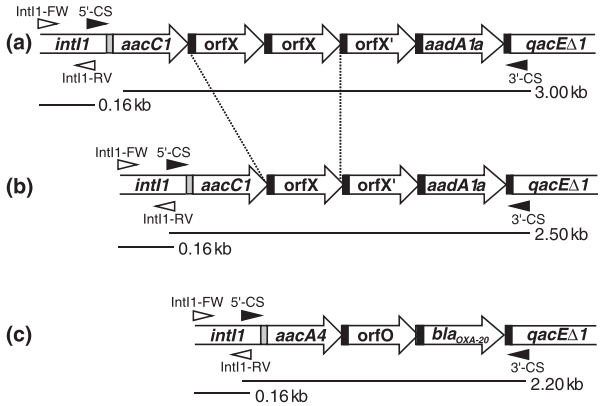


FIG. 4 Comparison of integron structures found in the prototypic European clone II and in *Acinetobacter baumannii* RAPD types 1 and 2. The structure of the 3.0-kb (a), 2.5-kb (b), and 2.2-kb (c) class 1 integrons detected in the *A. baumannii* European clone II (strain RUH134) and RAPD types 2 and 1, respectively, was inferred from DNA sequence analysis. Coding sequences (not to scale) are indicated by arrows and named according to ref. [44]; the *attC* (recombination site) and *attI* (attachment site) sites are shown as black and gray rectangles, respectively. Arrowheads indicate the position and direction of primers used in various combinations for PCR analysis. Horizontal lanes indicate the size of PCR products. The dotted line denotes the possible event of orfX insertion/deletion.

an AAD(3')-Ia aminoglycoside adenyltransferase. Intriguingly, the 3.0 kb integron of strain RUH134 showed substantial similarity with the 2.5 kb integron found in type 2 isolates (Fig. 4). No homology with known resistance genes was observed for the 0.75-kb amplicon sequence, which probably originates from mispriming of oligonucleotides 5′-CS and 3′-CS.

The antibiotic resistance genes found in the 2.2 and 2.5 kb integrons partly explain the antibiotic resistance profiles observed for type 1 and 2 *A. baumannii* strains. The presence of the *aacA4* genes in the 2.2 kb integron was associated with resistance to AMK, while the presence of the *aacC1* gene in the 2.5 kb integron was associated with resistance to GEN. However, the absence of these integron cassettes did not imply susceptibility to AMK and GEN.

A search for carbapenem resistance determinants showed that all isolates were negative for both bla_{OXA-23} and bla_{IMP} genes, and positive for the bla_{OXA-51} gene, irrespective of their resistance or susceptibility to carbapenems. The presence of the IS*Aba1* element upstream of the bla_{OXA-51} gene was not detected in any isolate. Seventy-one carbapenem-resistant *A. baumannii* isolates, including 70 RAPD-1 isolates and one RAPD-4 isolate, were positive for the bla_{OXA-58} gene, while all carbapenem-susceptible *A. baumannii* isolates, mostly belonging to the RAPD-2 type, were not. The bla_{OXA-24} gene was detected in the single *A. baumannii* isolate belonging to RAPD type 1a (study code 50). This strain differed from all other carbapenem-resistant *A. baumannii* isolates in that it was very high-level resistant to IPM (128 mg/L vs. 16-32 mg/L).

Hospital distribution of *A. baumannii* type 1 and type 2 isolates

Molecular typing revealed the prevalence of two epidemic clones accounting for 98.9% of all *A. baumannii* isolates (Fig. 5), as also documented for several *A. baumannii* outbreaks in Europe [37]. The dominant *A. baumannii* clone, referred to as type 1, was isolated from all hospitals (A–G) and accounted for 80.7% of isolates (Fig. 5). Type 1 strains were also isolated from the ICU environment associated with infection. Notably, both *A. baumannii* index strains from previous ICU outbreaks in Rome (hospitals F and G; [19,20]) belonged to type 1. The present investigation also shows that type 1 strains have been isolated from patients cared for in the ICU of hospital F several months before the onset of the outbreak (July–September 2005) [19], raising serious concern as to the persistence of this epidemic strain in the entire Rome urban area. The second *A. baumannii* clone, referred to as type 2, accounted for 18.2% of isolates (Fig. 5), and prevailed in hospital D (11 of 17 isolates) while coexisting with type 1 in hospital C (five of 37 isolates). Temporal clustering was noted in hospitals A, B, and C during the warmer season (May–October 2004; Fig. 5).

Discussion

Understanding the global epidemiology of *A. baumannii* infection has become a public health priority since MDR epidemic clones spread in several western countries and community-acquired *A. baumannii* pneumonia emerged as a novel clinical entity [1,2]. This calls for surveillance on both a national and international scale. Although *A. baumannii* strains related to European clones I and II have sporadically been documented in Italian hospitals [8,10,11,13], no systematic survey of *A. baumannii* epidemiology is available from Italy. Therefore, the molecular epidemiology and genetic basis of antibiotic resistance was investigated in a representative collection of MDR *A. baumannii* strains isolated in Rome during an 18-month period.

Since commercial systems can occasionally fail in *A. baumannii* identification, all isolates were first investigated at the genospecies level by ARDRA [24], and a satisfactory rate (97.8%) of species identification was found by collaborating centres. Interestingly, ARDRA differentiated *A. baumannii* into two main groups, accounting for 81.8% (type '11123') and 18.2% (type '11121') of *A. baumanni* isolates.

Here, evidence that MDR *A. baumannii* strains isolated from ICUs in Rome belong to two predominant types, genetically related to the European clones I and II is provided. In fact, the generation of similar DNA banding patterns between *A. baumannii* types 1 and 2 and the European

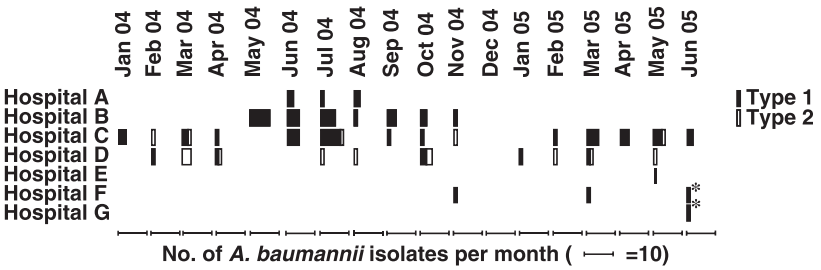


FIG. 5 Hospital distribution of *A. baumannii* clinical and environmental isolates during the 18-month investigation period. Asterisks denote *A. baumanii* index strains [19,20].

clones II and I, respectively, strongly argues for their genetic similarity. Accordingly, the same sequence group was determined for the European clone II and all type 1 strains, as well as for the European clone I and all type 2 strains. Minor differences (concerning <2 bands) in the RAPD fingerprints were observed, plausibly arising from ongoing diversification in space and time from the reference strains RUH134 (European clone II) and RUH875 (European clone I) which were both isolated in early 1980s [6]. Likewise, the criterion applied to correlate PFGE types (<6 band difference [29]) is stringent enough to ensure the genetic relatedness. Thus, the study isolates may represent a subgroup within the European clonal lineages I and II, as similarly suggested by Da Silva *et al.* [38] for Portuguese MDR *A. baumannii* isolates. Notably, the propensity of *A. baumannii* to evolve through extensive genome rearrangement is corroborated by genomic studies showing the ability of *A. baumannii* to acquire exogenous DNA, primarily carrying pathogenicity and drug-resistance islands [13,39–41]. Horizontal gene transfer events could result in diversification of molecular fingerprints, as suggested by this study.

Considering the broad spectrum of antibiotic resistance observed among MDR *A. baumannii*, it is worth noticing that a fairly high percentage of *A. baumannii* isolates belonging to both type 1 (48.6%) and type 2 (71.4%) was susceptible to the combination of ampicillin and sulbactam, in spite of the high overall resistance to other β-lactams. As previously demonstrated by Corbella *et al.* [42], the inhibitory effect of SAM on MDR *A. baumannii* is likely to be attributed to the activity of sulbactam alone, independent of β-lactamase inhibition. Hence, the present data suggest that SAM should be taken into account in the choice of commercial cards for use in *A. baumannii* identification and susceptibility testing, especially when alternative therapeutic options are needed to treat carbapenem-resistant *A. baumannii* infections.

There has been considerable concern regarding the recent reports of nosocomial outbreaks of carbapenem-resistant *A. baumannii* in Rome [18–20], and several studies documented the emergence of the carbapenem-hydrolysing β-lactamase $bla_{\text{OXA-58}}$ gene in clinical *A. baumannii* isolates [18,19,34]. The present findings demonstrate that the $bla_{\text{OXA-58}}$ gene accounts for MEM and IPM resistance in 98.6% of the carbapenem-resistant *A. baumannii* type 1 isolates, irrespective of the hospital source. Moreover, the single CS-resistant isolate, classified as RAPD-1a type, carried the $bla_{\text{OXA-24}}$ gene and showed high-level resistance to IPM. This strain is likely to be derived from the MDR type 1 lineage through evolution towards pan-resistance, resulting in susceptibility to TIG only. Overall, TIG resistance was observed for 22.8% and 50% of *A. baumannii* isolates belonging to types 1 and 2, respectively. This result is worrying, since TIG has recently been introduced in the Italian market and all isolates were obtained from patients who had never been treated with TIG. Moreover, individual type 1 and type 2 *A. baumannii* isolates displayed high variability with regard to TIG susceptibility, likely reflecting intraclonal diversification at the level of the presence and/or expression of TIG resistance mechanisms, such as efflux pumps [43].

The high rate of carbapenem and TIG resistance and the risk of emergence of pan-resistance among isolates related to the European epidemic clone II deserve consideration in clinical practice, infection surveillance, and hospital policy of antibiotic administration.

Further insight into the antimicrobial resistance determinants in *A. baumannii* isolates was provided by characterization of integrons, which were detected in 98.6% and 87.5% of type 1 and 2 strains, respectively, being absent only in RAPD-1a and RAPD-2a variants. However, a different array of gene cassettes was observed in *A. baumannii* integrons, with type 1 and type 2 isolates carrying the 2.2 and 2.5 kb variable regions, respectively. The gene content of both integrons is predicted to account for different aminoglycoside resistance patterns. Similar gene cassette arrays were previously identified in class 1 integrons of epidemic *A. baumannii* strains from different European countries [11,37,44]. Although integrons of *A. baumannii* outbreak strains appear to host a limited number of gene cassette arrangements, isolates of the same genotype may not only be associated with different integrons, but unrelated isolates of different genotypes may also contain the same integron [11,32,44]. Integron rearrangement, or exchange, can be imagined to explain the structural similarity observed between the 3.0 kb integron of the prototypic European clone II strain RUH134 and the 2.5 kb integron of type 2 strains, which are indeed related to the European clone I, rather than clone II. In fact, the 3.0-kb integron found in the prototypic European clone II (emerged in the early 1980s [11]) could generate the 2.5-kb integron (emerged in the late 1990s [44]) by excision of one the duplicate OrfX cassettes (Fig. 4).

This process could have occurred either by integrase-mediated or by homologous recombination, leading to loss of one of the duplicate cassettes in the ancestor integron.

Since strains belonging to both clonal lineages can coexist in the same hospital [45], it was hypothesized that horizontal transfer of entire integron structures between lineages may account for the variability of integrons carried by genetically related strains.

In conclusion, evidence of the emergence of two epidemic MDR *A. baumannii* clones, genetically related to the European clones I and II and responsible for infections in ICUs in the

main hospitals of the Rome urban area is provided. Although important questions concerning the origin and the 'epidemic success' of these clones remain unanswered, it must be emphasized that *A. baumannii* isolates of type I were found in all hospitals. Early recognition of these epidemic clones is therefore recommended in order to devise effective prevention and control measures for *A. baumannii* infection in ICUs.

Acknowledgements

We thank K. Towner (University Hospital, Public Health Laboratory, Nottingham, UK) for providing *A. baumannii* RUH875 and RUH134, and A. Cassone (Istituto Superiore di Sanità, Rome, Italy) for providing the two *A. baumannii* index strains.

Transparency Declaration

This work was supported by Ricerca Corrente grants from the Italian Ministry of Health to P. V. The authors declare no relationship or any degree of conflicting or dual interest, financial or of any other nature, that may affect professional judgment in relation to this article.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. ARDRA of representative *Acinetobacter* strains.

Fig. S2. RAPD fingerprints generated with primers M13 (A), ERIC-2 (B), and DAF4 (C) for 90 *Acinetobacter* isolates.

Fig. S3. PCR and RFLP analysis of integrons in RAPD-1 and RAPD-2 type *A. baumannii* strains.

Table S1. Sizing of restriction fragments generated from *Hin*fI digestion of the variable region of *A. baumannii* class 1 integrons.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

1. Munoz-Price LS, Weinstein RA. *Acinetobacter* infection. *N Engl J Med* 2008; 358: 1271–1281.
2. Dijkshoorn L, Nemec A, Seifert H. An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nat Rev Microbiol* 2007; 5: 939–951.
3. Abbo A, Navon-Venezia S, Hammer-Muntz O *et al.* Multidrug-resistant *Acinetobacter baumannii*. *Emerg Infect Dis* 2005; 11: 22–29.
4. Coelho JM, Turton JF, Kaufmann ME *et al.* Occurrence of carbapenem-resistant *Acinetobacter baumannii* clones at multiple hospitals in London and Southeast England. *J Clin Microbiol* 2006; 44: 3623–3627.
5. Dijkshoorn L, Aucken H, Gerner-Smidt P *et al.* Comparison of outbreak and nonoutbreak *Acinetobacter baumannii* strains by genotypic and phenotypic methods. *J Clin Microbiol* 1996; 34: 1519–1525.
6. Nemec A, Dijkshoorn L, van der Reijden TJ. Long-term predominance of two pan-European clones among multi-resistant *Acinetobacter baumannii* strains in the Czech Republic. *J Med Microbiol* 2004; 53: 147–153.
7. Nemec A, Janda L, Melter O *et al.* Genotypic and phenotypic similarity of multiresistant *Acinetobacter baumannii* isolates in the Czech Republic. *J Med Microbiol* 1999; 48: 287–296.
8. Brisse S, Milatovic D, Fluit AC *et al.* Molecular surveillance of European quinolone-resistant clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter* spp. using automated ribotyping. *J Clin Microbiol* 2000; 38: 3636–3645.
9. Spence RP, van der Reijden TJ, Dijkshoorn L *et al.* Comparison of *Acinetobacter baumannii* isolates from United Kingdom hospitals with predominant Northern European genotypes by amplified-fragment length polymorphism analysis. *J Clin Microbiol* 2004; 42: 832–834.
10. van Dessel H, Dijkshoorn L, van der Reijden T *et al.* Identification of a new geographically widespread multiresistant *Acinetobacter baumannii* clone from European hospitals. *Res Microbiol* 2004; 155: 105–112.
11. Nemec A, Dolzani L, Brisse S *et al.* Diversity of aminoglycoside-resistance genes and their association with class 1 integrons among strains of pan-European *Acinetobacter baumannii* clones. *J Med Microbiol* 2004; 53: 1233–1240.
12. Fournier PE, Richet H. The epidemiology and control of *Acinetobacter baumannii* in health care facilities. *Clin Infect Dis* 2006; 42: 692–699.
13. Iacono M, Villa L, Fortini D, *et al.* Whole genome pyrosequencing of an epidemic multidrug resistant Acinetobacter baumannii of the European clone II. *Antimicrob Agents Chemother* 2008; 52: 2616–2625.
14. Koeleman JG, Stoof J, Van Der Bijl MW *et al.* Identification of epidemic strains of *Acinetobacter baumannii* by integrase gene PCR. *J Clin Microbiol* 2001; 39: 8–13.
15. Villari P, Iacuzio L, Vozzella EA *et al.* Unusual genetic heterogeneity of *Acinetobacter baumannii* isolates in a university hospital in Italy. *Am J Infect Control* 1999; 27: 247–253.
16. Zarrilli R, Crispino M, Bagattini M *et al.* Molecular epidemiology of sequential outbreaks of *Acinetobacter baumannii* in an intensive care unit shows the emergence of carbapenem resistance. *J Clin Microbiol* 2004; 42: 946–953.
17. Agodi A, Zarrilli R, Barchitta M *et al.* Alert surveillance of intensive care unit-acquired *Acinetobacter* infections in a Sicilian hospital. *Clin Microbiol Infect* 2006; 12: 241–247.
18. Giordano A, Varesi P, Bertini A *et al.* Outbreak of *Acinetobacter baumannii* producing the carbapenem-hydrolyzing oxacillinase OXA-58 in Rome, Italy. *Microb Drug Resist* 2007; 13: 37–43.
19. Bertini A, Giordano A, Varesi P *et al.* First report of the carbapenem-hydrolyzing oxacillinase OXA-58 in *Acinetobacter baumannii* isolates in Italy. *Antimicrob Agents Chemother* 2006; 50: 2268–2269.
20. Longo B, Pantosti A, Luzzi I *et al.* An outbreak of *Acinetobacter baumannii* in an intensive care unit: epidemiological and molecular findings. *J Hosp Infect* 2006; 64: 303–305.
21. Clinical and Laboratory Standard Institute. *Performance standards for antimicrobial susceptibility testing*, Seventeenth informational supplement. M100-S17. Wayne, PA: CLSI, 2007.

22. Gales AC, Reis AO, Jones RN. Contemporary assessment of antimicrobial susceptibility testing methods for polymyxin B and colistin: review of available interpretative criteria and quality control guidelines. *J Clin Microbiol* 2001; 39: 183–190.
23. Paterson DL. The epidemiological profile of infections with multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter* species. *Clin Infect Dis* 2006; 43 (suppl): 43S–48S.
24. Dijkshoorn L, Van Harsselaar B, Tjernberg I *et al.* Evaluation of amplified ribosomal DNA restriction analysis for identification of *Acinetobacter* genomic species. *Syst Appl Microbiol* 1998; 21: 33–39.
25. Vaneechoutte M, Dijkshoorn L, Tjernberg I *et al.* Identification of *Acinetobacter* genomic species by amplified ribosomal DNA restriction analysis. *J Clin Microbiol* 1995; 33: 11–15.
26. Grundmann HJ, Towner KJ, Dijkshoorn L *et al.* Multicenter study using standardized protocols and reagents for evaluation of reproducibility of PCR-based fingerprinting of *Acinetobacter* spp. *J Clin Microbiol* 1997; 35: 3071–3077.
27. Carr E, Eason H, Feng S *et al.* RAPD-PCR typing of *Acinetobacter* isolates from activated sludge systems designed to remove phosphorus microbiologically. *J Appl Microbiol* 2001; 90: 309–319.
28. Seifert H, Dolzani L, Bressan R *et al.* Standardization and interlaboratory reproducibility assessment of pulsed-field gel electrophoresis-generated fingerprints of *Acinetobacter baumannii*. *J Clin Microbiol* 2005; 43: 4328–4335.
29. Tenover FC, Arbeit RD, Goering RV *et al.* Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; 33: 2233–2239.
30. Struelens MJ, Schwam V, Deplano A *et al.* Genome macrorestriction analysis of diversity and variability of *Pseudomonas aeruginosa* strains infecting cystic fibrosis patients. *J Clin Microbiol* 1993; 31: 2320–2326.
31. Turton JF, Gabriel SN, Valderrey C *et al.* Use of sequence-based typing and multiplex PCR to identify clonal lineages of outbreak strains of *Acinetobacter baumannii*. *Clin Microbiol Infect* 2007; 13: 807–815.
32. Ploy MC, Denis F, Courvalin P *et al.* Molecular characterization of integrons in *Acinetobacter baumannii*: description of a hybrid class 2 integron. *Antimicrob Agents Chemother* 2000; 44: 2684–2688.
33. Sambrook JE, Fritsch F, Maniatis T. *Molecular cloning: a laboratory manual*, 2th edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
34. Poirel L, Marque S, Heritier C *et al.* OXA-58, a novel class D {beta}-lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2005; 49: 202–208.
35. Woodford N, Ellington MJ, Coelho JM *et al.* Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. *Int J Antimicrob Agents* 2006; 27: 351–353.
36. Turton JF, Ward ME, Woodford N *et al.* The role of ISAba1 in expression of OXA carbapenemase genes in *Acinetobacter baumannii*. *FEMS Microbiol Lett* 2006; 258: 72–77.
37. Turton JF, Kaufmann ME, Glover J *et al.* Detection and typing of integrons in epidemic strains of *Acinetobacter baumannii* found in the United Kingdom. *J Clin Microbiol* 2005; 43: 3074–3082.
38. Da Silva G, Dijkshoorn L, van der Reijden T *et al.* Identification of widespread, closely related *Acinetobacter baumannii* isolates in Portugal as subgroup of European clone II. *Clin Microbiol Infect* 2007; 13: 190–195.
39. Fournier PE, Vallenet D, Barbe V *et al.* Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *PLoS Genet* 2006; 2: e7.
40. Smith MG, Gianoulis TA, Pukatzki S *et al.* New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. *Genes Dev* 2007; 21: 601–614.
41. Valenzuela JK, Thomas L, Partridge SR *et al.* Horizontal gene transfer in a polyclonal outbreak of carbapenem-resistant *Acinetobacter baumannii*. *J Clin Microbiol* 2007; 45: 453–460.
42. Corbella X, Ariza J, Ardanuy C *et al.* Efficacy of sulbactam alone and in combination with ampicillin in nosocomial infections caused by multiresistant *Acinetobacter baumannii*. *J Antimicrob Chemother* 1998; 42: 793–802.
43. Peleg AY, Adams J, Paterson DL. Tigecycline efflux as a mechanism for nonsusceptibility in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2007; 51: 2065–2069.
44. Gombac F, Riccio ML, Rossolini GM *et al.* Molecular characterization of integrons in epidemiologically unrelated clinical isolates of *Acinetobacter baumannii* from Italian hospitals reveals a limited diversity of gene cassette arrays. *Antimicrob Agents Chemother* 2002; 46: 3665–3668.
45. Towner KJ, Levi K, Vlassiadi M *et al.* Genetic diversity of carbapenem-resistant isolates of *Acinetobacter baumannii* in Europe. *Clin Microbiol Infect* 2008; 14: 161–167.