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EPIDEMIOLOGIA MOLECOLARE DI *Acinetobacter baumannii* MULTIRESISTENTE IN UNITÀ DI TERAPIA INTENSIVA DI OSPEDALI DEL SUD EUROPA

MOLECULAR EPIDEMIOLOGY OF MULTI-DRUG RESISTANT *Acinetobacter baumannii* IN INTENSIVE CARE UNITS OF MULTIPLE SOUTH EUROPEAN HOSPITALS

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CHAPTER 1

INTRODUCTION

Acinetobacter baumannii

Acinetobacter spp. are glucose-non fermentative gram-negative, strictly aerobic, non-motile, oxidasenegative, catalase-positive coccobacilli that have been increasingly associated with severe infections [1,2].

The existence of the genus *Acinetobacter* has been described initially in 1954, in one study [3], followed by many changes that led in 1986 to the current classification system [4]**.** Based on more recent taxonomic data, it was proposed that members of the genus *Acinetobacter* should be classified in the new family *Moraxellaceae.* Using DNA-DNA hybridization technique have been described so far 32 differentiated species, of which 17 have been named [5-7]. (TABLE 1) *Acinetobacter* gen. sp 3 and 13TU are also pathogenic in human beings although they are less frequeltly encountered [8,9]. These three species are closely related genetically, and cannot be accurately differentiated by routine phenotypic methods; and it has been proposed that they should be grouped together into the so-called *A. calcoaceticus-A. baumannii* (Acb) complex [1].

A. baumannii is ubiquitous in nature and has been recovered from soil, water, animals, and humans. *Acinetobacter* species are normal inhabitants of human skin and are frequently isolated from the throat and respiratory tract of hospitalized patients. For this reason, it has been suggested that human skin could be the source of severe infections, such as bacteremia. However, this hypothesis has not been confirmed in healthy humans. [10]

A. baumannii has emerged in the last few decades as a major cause of healthcare-associated infections (HAIs) and nosocomial outbreaks [2].

Table 1 Classification of the genus *Acinetobacter*

Species Source References	Species Source References
Species that have valid name	
Acinetobacter calcoaceticus	Soil and humans (including clinical specimens)
Acinetobacter baumannii	Humans (including clinical specimens), soil, meat and Vegetables
Acinetobacter haemolyticus	Humans (including clinical specimens)
Acinetobacter junii	Humans (including clinical specimens)
Acinetobacter johnsonti	Humans (including clinical specimens) and animals
Acinetobacter lwoffii **(including gen.sp. 9)**	Humans (including clinical specimens) and animals
Acinetobacter radioresistens	Humans (including clinical specimens), soil and cotton
Acinetobacter ursingii	Humans (including clinical specimens)
Acinetobacter schindleri	Humans (including clinical specimens)
Acinetobacter parvus	Humans (including clinical specimens) and animals
Acinetobacter baylyi	Activated sludge and soil
Acinetobacter bouvetii	Activated sludge
Acinetobacter towneri	Activated sludge
Acinetobacter tandoii	Activated sludge
Acinetobacter grimontii	Activated sludge
Acinetobacter tjernbergiae	Activated sludge
Acinetobacter gerneri	Activated sludge
Species that have provisional designations	
Acinetobacter venetianus	Sea water
Gen.sp. 3	Humans (including clinical specimens), soil and vegetables
Gen.sp. 6	Humans (including clinical specimens),
Gen.sp. 10	Humans (including clinical specimens), soil and vegetables
Gen.sp. 11	Humans (including clinical specimens), soil and animals
Gen.sp. 13BJ or 14TU*	Humans (including clinical specimens)
Gen.sp. 14BJ	Humans (including clinical specimens)
Gen.sp. 15BJ	Humans (including clinical specimens)
Gen.sp. 16	Humans (including clinical specimens) and vegetables
Gen.sp. 17	Humans (including clinical specimens) and soll
Gen.sp. 13TU	Humans (including clinical specimens)
Gen.sp. 15TU	Humans (including clinical specimens)
Gen.sp. 'between 1 and 3'	Humans (clinical specimens
Gen.sp. 'close to 13TU'	Humans (clinical specimens

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Acinetobacter species identification

DNA-DNA hybridization remains the reference standard for identification of *Acinetobacter* species, [4]. The phenotypic identification scheme proposed by Bouvet and Grimont in 1986 is based on 28 phenotypic tests [4]. This identification scheme was refined in 1987 by the same authors and includes growth at 37°C, 41°C, and 44°C; production of acid from glucose; gelatin hydrolysis; and assimilation of 14 different carbon sources [3]. In particular, the closely related and clinically most relevant species *A. baumannii* and *Acinetobacter* genomic species 13TU cannot be distinguished, while *A. calcoaceticus* and *Acinetobacter* genomic species 3 can only be separated by their growth properties at different temperatures [12]. Genotyping allows investigation of clonal spread and can be used to identify the source of the original infection. Traditional *Acinetobacter* strain typing methods include serotyping [13], multilocus enzyme electrophoresis [14] and DNA-based methods, including repetitive extragenic palindromic sequence-based PCR [15,16], amplified ribosomal DNA restriction analysis (ARDRA), pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) [17] and ribotyping [18,19,20].
However, these methods are not optimal: despite strenuous efforts at standardization, it is difficult to compare results obtained in different laboratories, and the methods are labor intensive and time consuming. Two other techniques have been proposed to characterize clinical isolates of *A. baumannii*, both based to amplification and sequence analysis. The first is the MultiLocus Sequence Typing (MLST), a technique that is a high-resolution molecular tool for discriminating between closely related bacterial species [21]. MLST data are reproducible and portable, facilitating comparison among laboratories worldwide. MLST is applicable to almost all bacterial species pathogenic,and allows to follow through comparison with a databases available on-line the global spread of strains with specific characteristics of virulence or antibiotic resistance [22]. The MLST provides an analysis of 7 housekeeping genes for *A. baumannii* and has

a high discriminative power [7]. The second technique, i.e. sequence-based typing (ST), allows the assignment of a particular strain of epidemic clones through amplification and sequencing of three genes, in particular *omp*A (outer membrane protein A), csuE (part of a pilus assembly system, thought to be essential for biofilm formation and $bla_{\text{OXA-51-like}}$ (intrinsic carbapenemase of *A. baumannii*). Only isolates belonging to epidemic clones have the same combination of alleles for the three genes [23].

Nosocomial infections

The propensity for outbreaks of multidrug-resistant *A. baumannii* has been demonstrated clearly. Depending on the local circumstances, and the strain in question, the pattern of an outbreak can vary. To cause an epidemic bacteria must be able getting into the environment and this takes place mainly in three ways: by air, contamination of materials and through transmission by hospital staff. Multidrug-resistant (MDR) *A. baumannii* is a rapidly emerging pathogen in the healthcare setting, where it causes infections that include bacteremia, pneumonia, meningitis, urinary tract infection and wound infection. *A.baumannii* infects hospitalized, immunocompromised, long hospital-staying patients subjected to prolonged antibiotic therapy and invasive procedures. The main sites of infection are the respiratory tract, urinary tract, blood and skin. Infections often involve hospitalized patients in intensive care unit (ICU) and are associated with a high level of mortality. A scheme that depicts the dynamics of epidemic *A. baumannii* on a hospital ward is provided in FIG. 2 [1].An epidemic strain is most commonly introduced by a patient who is colonized. Once on a ward, the strain can then spread to other patients and their environment. The main characteristic of *A. baumannii* is the capability of surviving for prolonged periods in the environment, thus contributing to the transmission of the organism during outbreaks [24]. *A. baumannii* isolates are able to survive for weeks under dry conditions and have

been detected on hospital bed rails until 9 days after the discharge of an infected patient, suggesting that hospital equipments could serve as a secondary reservoir for the infection [25]. During outbreaks *A. baumannii* has been recovered from various sites in the patients' environment, including bed curtains, furniture and hospital equipment [26]. The bacteria can be spread through the air over short distances in water droplets and in scales of skin from patients who are colonized [27]. The transfer *A. baumannii* from hospital staff to patients appears to be an important means for spread of epidemics [2].

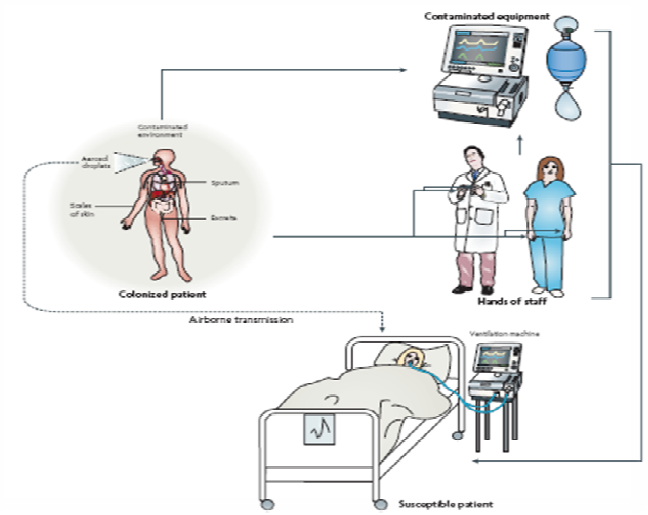

Figure 2 | **Overview of the dynamics between patients, bacteria and the hospital environment.** The possible modes of Acinetobacter baumannii entry into a ward are shown.

- Entrance through a colonized patient is the most likely mode.
- Introduction through contaminated materials [28]
- Introduction by healthy carriers.

The acquisition of *A. baumannii* by susceptible patients can occur through various routes, of which the hands of hospital staff are thought to be the most common, although the precise mode of transmission is usually difficult to assess.

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Pathogenicity and virulence factors

In the past, *Acinetobacter* was considered to be an organism of low virulence. The occurrence of fulminant community' acquired *Acinetobacter* pneumonia indicates that these organisms may sometimes be of high pathogenicity and cause invasive disease. Studies on *Acinetobacter* virulence factors are still at an elementary stage. A number of putative mechanisms that might have a role in colonization,

infection and epidemic spread are summarized in FIG. 3 [1]. Genetic, molecular and experimental studies are required to elucidate these mechanisms in more detail.

Recent DNA sequencing of a single *A. baumannii* strain identified 16 genomic islands that carry putative virulence genes that are associated with, for example, cell-envelope biogenesis, antibiotic resistance, autoinducer production, pilus biogenesis and lipid metabolism [30]. Resistance to desiccation, disinfectants [29,31] and antibiotics is important for environmental survival. The extraordinary metabolic versatility of *A. baumannii* could contribute to its proliferation on a ward and in patients. Pilus-mediated biofilm formation on glass and plastics has been demonstrated [32]. If formed on medical devices, such as endotracheal tubes or intravascular catheters, these biofilms would probably provide a niche for the bacteria, from which they might colonize patients and give rise to respiratory-tract or bloodstream infections. Electron microscopy studies have demonstrated that pili on the surface of acinetobacters interact with human epithelial cells [33]. In addition, thread-like connections between these bacteria were suggestive of an early phase of biofilm formation. The pili and hydrophobic sugars in the O-side-chain moiety of lipopolysaccharide (LPS) [34] might promote adherence to host cells as a first step in the colonization of patients. *In vitro* and animal experiments have identified various factors that could have a role in *A. baumannii* infection. For example, *A. baumannii* outer membrane protein A (AbOmpA, previously called Omp38) has been associated with the induction of cytotoxicity [35]. Iron-acquisition mechanisms [36] and serum resistance [37] are attributes that enable the organism to survive in the bloodstream.

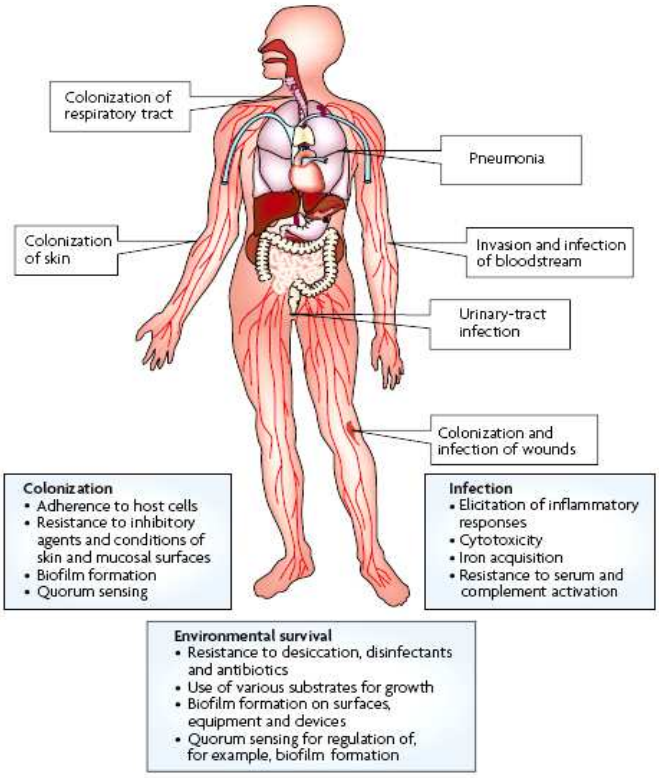

Figure 3 The factors that contribute to Acinetobacter baumannii environmental persistence and host infection and colonization. Adherence to host cells, as demonstrated in an in vitro model using bronchial epithelial cells [33], is considered to be a first step in the colonization process. Survival and growth on host skin and mucosal surfaces require that the organisms can resist antibiotics and inhibitory agents and the conditions that are exerted by these surfaces. Outgrowth on mucosal surfaces and medical devices, such as intravascular catheters and endotracheal tubes [32], can result in biofilm formation, which enhances the risk of infection of the bloodstream and airways. Quorum sensing [30] might have a regulatory role in biofilm formation. Experimental studies have identified various factors that could have a role in *A. baumannii* infection, for example, lipopolysaccharide has been shown to elicit a proinflammatory response in animal models [38,39]. Furthermore, the *A. baumannii* outer membrane protein A has been demonstrated to cause cell death in vitro [35]. Iron-acquisition mechanisms [36] and resistance to the bactericidal activity of human serum [37] are considered to be important for survival in the blood during bloodstream infections. Environmental survival and growth require attributes such as resistance to desiccation [29,31] versatility in growth requirements [2], biofilmformingcapacity [32] and, probably, quorum-sensing activity [30]. Finally, adequate stressresponse mechanisms are thought to be required for adaptation to different conditions

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Antibiotic resistance in *Acinetobacter baumannii*

Acinetobacter infections have become more difficult to treat owing to the emergence of isolates resistant to all commonly prescribed antimicrobial

drugs [40]. The acquisition of resistance mechanisms by *A. baumannii* has been estimated as a recent phenomenon that started in the 1970s [41]. Resistance mechanisms involve antimicrobial-degrading enzymes, efflux pumps, target modification and porin deficiency [42]. The acquisition of a MDR phenotype can be caused by mobile genetic elements (plasmids, transposons and integrons) [43-47]. This extremely rapid development of antimicrobial resistance is due to the widespread use of antimicrobials in the hospital environment and to the ability of *A. baumannii* to respond rapidly to challenges issued by antimicrobials [48]. The *Acinetobacter* have become resistant to almost all antibiotics available, including aminoglycosides, quinolones and extended-spectrum beta-lactam antibiotics. Most strains are resistant to cephalosporins, whereas resistance to carbapenems is increasingly reported frequently [49]. The percentages of resistance are generally higher for blocks of intensive care unit (ICU), particularly those epidemics [49,50].For pan-resistant isolates, colistin has been used with success in severe infections, including meningitis, bacteremia or pneumonia [51-53]. More recently, the use of colistin has been associated with lower rates of renal toxicity, suggesting that the incidence and severity of systemic toxicity owing to colistin administration was probably overstated [54-56].

CHAPTER 2

Identification of *Acinetobacter* Genomic Species 13TU by Sequence Analysis of the 16S-23S rRNA Gene Spacer Region

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The genus *Acinetobacter* currently contains up to 32 described named and unnamed (genomic) species [1]. *Acinetobacter baumannii*, genomic species 3, and 13TU, three of the most clinically relevant species, are genetically and phenotypically very similar to an environmental species, *Acinetobacter calcoaceticus*, and are therefore grouped together into the socalled *A. calcoaceticus-A.baumannii* (Acb) complex [1].

Because phenotypic identification of *Acinetobacter* isolates to the species level has proven to be insufficient, several genotypic methods have been developed for genomic species identification [1,57,59]. In the last 3 years, our laboratory has routinely used the 16S-23S rRNA gene intergenic spacer (ITS) sequence-based method described by Chang et al. [57] to delineate isolates belonging to the Acb complex to the genomic species level. We found the procedure accurate and easy to perform. However, we have recently encountered a discrepancy during the characterization of *Acinetobacter* strain 74510 isolated originally from a clinical source at the Prince of Wales Hospital, Hong Kong, in 1995 [58]. Strain 74510 was previously identified as genomic species 13TU by restriction analysis of amplified ribosomal DNA [58,59]. However, examination of the single PCR amplicon of the ITS region of strain 74510 (GenBank accession no. FJ360743) revealed an ITS sequence length of 607 bp, as opposed to the 615 bp reported by Chang et al. for the genomic species 13TU reference strain BCRC 15417 [57]. On the other hand, DNA sequence analysis of this 607-bp ITS of strain 74510 showed an identity of 0.9561 to that of strain BCRC 15417 (Table 1). Upon further analysis of nucleotide sequences in GenBank, the strain 74510 ITS sequence was also found to share increasing identity (0.9950 to 0.9967) with three other genomic species 13TU strains, namely, v104-2, 00574, and DR25612/96 (Table 1). Interestingly, the ITS length (607 bp) of these three genomic species 13TU strains was also 607 bp, identical to that of strain 74510 (Table 1). In addition, the ITS sequences of these genomic species 13TU strains 74510, v104-2, 00574, and DR25612/96 appeared to be more closely related to the *A. baumannii* (genomic species 2) strain BCRC 10591T than to the 13TU reference strain BCRC 15417 (0.9538 to 0.9588

versus 0.9171 identity) (Table 1), which reinforces the notion that genetic similarity among *A. baumannii* and genomic species 13TU is high [1] and perhaps suggests that BCRC 15417 may not be as representative for genomic species 13TU.

Acinetobacter genomic species 13TU is the second most clinically relevant species in the Acb complex [1,60,61]. Therefore, we believe that it is important to extend the number of *Acinetobacter* genomic species 13TU ITS sequences in the ITS sequence database for more-accurate genomic species identification. Also, we would suggest that the reference ITS sequence length for genomic species 13TU be 607 bp long and that strain 74510 could be considered as the "type" strain for genomic species 13TU.

TABLE 1. ITS sequence similarity between *A. baumannii* and genomic species 13TU[a]

Acinetobacter strain	GenBank accession no.	ITS length (bp)	Sequence similarity with the indicated *Acinetobacter* strains				
			A. baumannii BCRC 10591^T	gsp 13TU			
				74510	v104-2	00574	DR25612/96	BCRC 15417
A. baumannii BCRC 10591^T	AY601824	607		0.9538	0.9555	0.9555	0.9588	0.9171
gsp 13TU 74510	FJ360743	607	0.9538		0.9950	0.9950	0.9967	0.9561
gsp 13TU v104-2	AY510071	607	0.9555	0.9950		0.9967	0.9950	0.9561
gsp 13TU 00574	AY510070	607	0.9555	0.9950	0.9967		0.9983	0.9528
gsp 13TU DR25612/96	EU030649	607	0.9588	0.9967	0.9950	0.9983		0.9512
gsp 13TU BCRC 15417	AY601830	615	0.9171	0.9561	0.9561	0.9528	0.9512	

[a] gsp, genomic species.

References are presented in the general reference list.

CHAPTER 3

Research Letter

Molecular epidemiology of carbapenem-resistant *Acinetobacter baumannii* strains in intensive care units of multiple Mediterranean hospitals

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Molecular epidemiology of carbapenem-resistant *Acinetobacter baumannii* strains in intensive care units of multiple Mediterranean hospitals

Sir,

Acinetobacter baumannii is an emerging opportunistic nosocomial pathogen in intensive care unit patients [62]. Outbreaks of carbapenem-resistant *A. baumannii* have been recently described in the Mediterranean [62-66]. The aim of the present study was to: (i) analyse the genetic relatedness of *A. baumannii* isolates associated with cross-transmission episodes that occurred in 18 hospitals in Greece, Italy, Lebanon and Turkey from 1999 to 2006; and (ii) identify the carbapenemase genes and their flanking IS elements. Twenty-four *A. baumannii* strains were included in the study. Antimicrobial susceptibilities were determined by a reference microdilution method [67]. *A. baumannii* strains exhibiting imipenem and/or meropenem MIC_8 mg/L were considered carbapenem-resistant. PFGE and dendrogram analysis was performed as reported previously [65]. The majority of the strains were isolated with identical PFGE type from more than two patients of the same or different institutions. Strain 3237 of PFGE type C was typed from one patient in Beirut, Lebanon; strains of PFGE types M and M1 were typed from three patients each, in three and two different Turkish hospitals, respectively (Table 1). Thirteen major PFGE types were identified, PFGE types A and M showing two subtypes and one subtype, respectively [Table 1 and Figure S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]. Multiplex PCR experiments [23] assigned 15 isolates to previously defined sequence type (ST) groups 1, 2 and 3, but were not able to identify the sequence group of 9 strains. Sequence-based typing was then performed using ompA, csuE and $bla_{\text{OXA-51-like}}$ sequences [23] These experiments confirmed the assignment of the strains of PFGE profiles A and B to ST group 1, strains of PFGE profiles D, E, G, H and I to ST group 2, and the strain of PFGE profile C to ST group 3. This is in accordance with previous reports showing that *A. baumannii* strains circulating in Europe

belonged to ST groups 1 and 2 that corresponded to the previously characterized European clones II and I, respectively [62,23]. Strains of ST group 1 with PFGE profile A predominated, being isolated in two hospitals in Naples, Italy, and in three Greek hospitals from 2002 to 2006, thus suggesting that the spread of this successful clone might have resulted from inter-hospital transfer of colonized patients within the same city or between different countries as described for other *A. baumannii* epidemics [62]. *A. baumannii* strains of six distinct PFGE types that were assigned to ST group 2 were isolated in Naples and Agrigento, Italy, in Thessaloniki, Athens and Serres, Greece, and in Beirut, Lebanon. Also, as already observed in northern Europe [62,23] a shift in the recent *A. baumannii* population towards ST group 1 (European clone II) was observed in Greece and Italy. In addition, ST analysis identified two novel ompA alleles, 5 and 6 (GenBank accession numbers EU426837 and EU426838), four novel csuE alleles, 7, 8, 8-1 and 9 (GenBank accession numbers EU426834– EU426836 and EU478794) and three novel *bla*$_{\text{OXA-51-like}}$ alleles, 6, 7 and 8 (GenBank accession numbers DQ149247, AJ309734 and EU375515) in isolates from Greece, Italy and Turkey. *ompA* allele 5 was 9 bp longer than other *ompA* alleles; *csuE* allele 8-1 differed from allele 8 reported by Turton et al. [23] by 1 nt. Two novel ST groups, 4 and 5, were assigned to strains showing allele profiles 4/7/4 and 5/8/7 or 5/8-1/7 at *ompA*/*csuE*/*bla*$_{\text{OXA-51-like}}$ loci, respectively. ST group 4 included strains of PFGE profile K isolated in Thessaloniki, Greece, and strains of PFGE profile L isolated in Kocaeli, Turkey. ST group 5 was assigned to several strains isolated in different Turkish cities. No novel ST group was assigned to strain 3866 of PFGE type F and strain 2977 of PFGE type N because they were micro-epidemic strains isolated from less than three patients. Interestingly, a peculiar distribution of *A. baumannii* strains of distinct ST groups was observed within the study countries. Thus, strains of ST groups 1 and 2, which were found all over Europe, [62,23] were frequently isolated in Greece, Italy and Lebanon. In contrast, strains of ST groups 4 and 5 were isolated within restricted geographical areas; strains of ST group 4 being isolated in Thessaloniki,

Greece, and Kocaeli, Turkey, and strains of ST group 5 being isolated in several Turkish cities (Table1). Molecular epidemiology of carbapenem resistance genes showed that 21 of 24 strains were carbapenem-resistant and produced OXA-58 carbapenemase, thus suggesting that the spread of *bla*$_{\text{OXA-58}}$ might have contributed to their selection. Other carbapenemase genes that might have selected *A. baumannii* strains included *bla*$_{\text{OXA-23}}$, found in strains of ST group 4 in Kocaeli, Turkey, as well as *bla*$_{\text{VIM-1}}$ and *bla*$_{\text{VIM-4}}$ metallo-b-lactamase genes, found in strains of ST groups 1 and 2, respectively, in Serres, Greece (Table 1).

Additional epidemiological information was provided by the analysis of the IS elements surrounding *bla*$_{\text{OXA-58}}$ [Table 1 and Figure S2, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]. In particular, the 5' end was flanked by either ISAba2, IS18 or an ISAba1 element, while an ISAba3 element at the 3' end of the gene occurred in all strains. Of note, each of the IS elements flanking the 5' end of *bla*$_{\text{OXA-58}}$ occurred in strains of distinct ST groups and PFGE profiles isolated in the same geographical area. Thus, the ISAba2 element was detected in Greece and Italy, IS18 in Lebanon and Turkey and ISAba1 in Turkey and Italy, suggesting that they might have been acquired through horizontal gene transfer. In partial support of this hypothesis, plasmid-borne *bla*$_{\text{OXA-58}}$ has been found in the majority of *A. baumannii* strains studied herein and in Acinetobacter spp. isolates in Europe [62,63–66,68]. Also, mobilization of IS elements by homologous recombination has been postulated,[68] and conjugative transfer of a plasmid-borne *bla*$_{\text{OXA-58}}$ gene flanked by IS18 and ISAba3 elements at the 5' and 3' ends, respectively, has been demonstrated [66].

In conclusion, results indicate that Mediterranean *A. baumannii* epidemics were sustained by the spread of distinct genotypes belonging to ST groups 1, 2, 4 and 5. The *bla*$_{\text{OXA-58}}$ gene flanked by IS elements was present in all carbapenem-resistant genotypes and possibly contributed to their selection.

Acknowledgements

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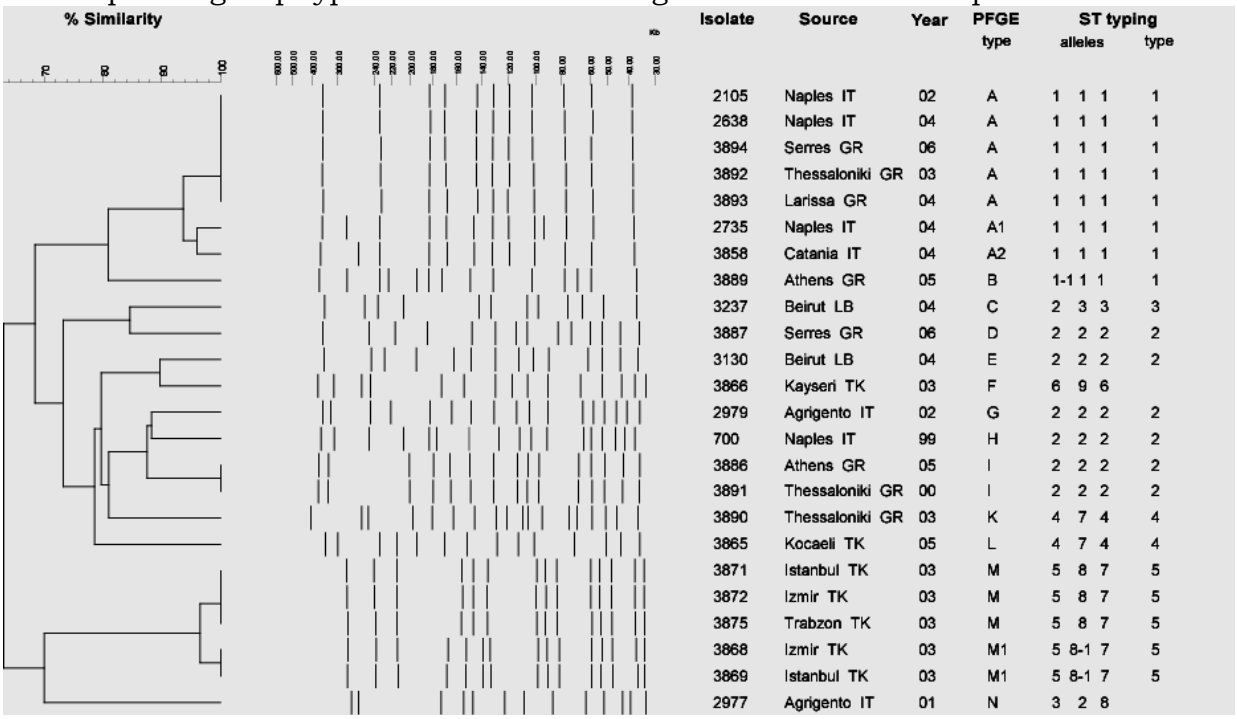
Table 1. Molecular epidemiology of *A. baumannii* strains included in this study

Strain	Hospital	Year	Patients[a]	PFGE type	Allelic profile			ST	Carbapenemase(s)	IS elements 5′–3′ to bla_{OXA-58}
					ompA	*csuE*	$bla_{OXA-51-like}$			
2105	Naples/IT	2002	43	A	1	1	1	1	OXA-58	IS*Aba2*/IS*Aba3*
2638	Naples/IT	2003	42	A	1	1	1	1	OXA-58	IS*Aba2*/IS*Aba3*
3894	Serres/GR	2006	19	A	1	1	1	1	OXA-58, VIM-1	IS*Aba2*/IS*Aba3*
3892	Thessaloniki/GR	2003	23	A	1	1	1	1	OXA-58	IS*Aba2*/IS*Aba3*
3893	Larissa/GR	2004	48	A	1	1	1	1	OXA-58	IS*Aba2*/IS*Aba3*
2735	Naples/IT	2004	2	A1	1	1	1	1	OXA-58	IS*Aba2*/IS*Aba3*
3858	Catania/IT	2004	27	A2	1	1	1	1	absent	
3889	Athens/GR	2005	4	B	1-1	1	1	1	OXA-58	IS*Aba2*/IS*Aba3*
3237	Beirut/LB	2004	1	C	2	3	3	3	absent	
3887	Serres/GR	2006	5	D	2	2	2	2	OXA-58, VIM-4	IS*Aba2*/IS*Aba3*
3130	Beirut/LB	2004	17	E	2	2	2	2	OXA-58	IS*18*/IS*Aba3*
3866	Kayseri/TK	2003	2	F	6	9	6		OXA-58	IS*Aba1*/IS*Aba3*
2979	Agrigento/IT	2002	14	G	2	2	2	2	absent	
700	Naples/IT	1999	81	H	2	2	2	2	absent	
3886	Athens/GR	2005	4	I	2	2	2	2	OXA-58	IS*Aba2*/IS*Aba3*
3891	Thessaloniki/GR	2000	3	I	2	2	2	2	OXA-58	IS*Aba2*/IS*Aba3*
3890	Thessaloniki/GR	2003	12	K	4	7	4	4	OXA-58	IS*Aba2*/IS*Aba3*
3865	Kocaeli/TK	2005	47	L	4	7	4	4	OXA-58, OXA-23	IS*Aba1*/IS*Aba3*
3871	Istanbul/TK	2003	1	M	5	8	7	5	OXA-58	IS*18*/IS*Aba3*
3872	Izmir/TK	2003	1	M	5	8	7	5	OXA-58	IS*18*/IS*Aba3*
3875	Trabzon/TK	2003	1	M	5	8	7	5	OXA-58	IS*Aba1*/IS*Aba3*
3868	Izmir/TK	2003	2	M1	5	8-1	7	5	OXA-58	IS*Aba1*/IS*Aba3*
3869	Istanbul/TK	2003	1	M1	5	8-1	7	5	OXA-58	IS*Aba1*/IS*Aba3*
2977	Agrigento/IT	2001	1	N	3	2	8		OXA-58	IS*Aba1*/IS*Aba3*

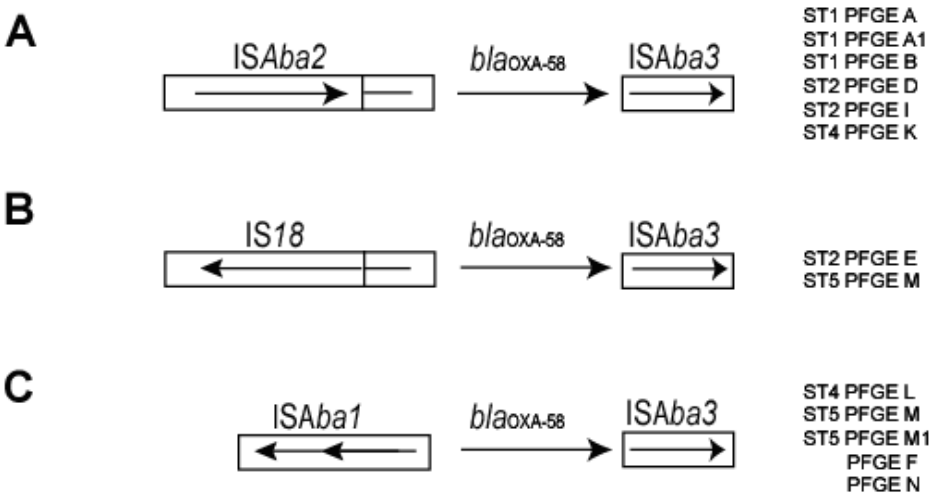
IT, Italy; GR, Greece; LB, Lebanon; TK, Turkey.
[a]Number of patients where each particular PFGE type was detected.

Figure S1. Genotype analysis of digitized *Apa*I PFGE profiles of *A. baumannii* strains included in the study. A percentage genetic similarity scale is shown above the dendrogram. Isolate number, source, year of isolation, PFGE type and sequence group type are shown on the right-hand side of each profile.



Isolate	Source	Year	PFGE type	ST typing alleles	ST type
2105	Naples IT	02	A	1 1 1	1
2638	Naples IT	04	A	1 1 1	1
3894	Serres GR	06	A	1 1 1	1
3892	Thessaloniki GR	03	A	1 1 1	1
3893	Larissa GR	04	A	1 1 1	1
2735	Naples IT	04	A1	1 1 1	1
3858	Catania IT	04	A2	1 1 1	1
3889	Athens GR	05	B	1-1 1 1	1
3237	Beirut LB	04	C	2 3 3	3
3887	Serres GR	06	D	2 2 2	2
3130	Beirut LB	04	E	2 2 2	2
3866	Kayseri TK	03	F	6 9 6	
2979	Agrigento IT	02	G	2 2 2	2
700	Naples IT	99	H	2 2 2	2
3886	Athens GR	05	I	2 2 2	2
3891	Thessaloniki GR	00	I	2 2 2	2
3890	Thessaloniki GR	03	K	4 7 4	4
3865	Kocaeli TK	05	L	4 7 4	4
3871	Istanbul TK	03	M	5 8 7	5
3872	Izmir TK	03	M	5 8 7	5
3875	Trabzon TK	03	M	5 8 7	5
3868	Izmir TK	03	M1	5 8-1 7	5
3869	Istanbul TK	03	M1	5 8-1 7	5
2977	Agrigento IT	01	N	3 2 8	

Figure S2. Schematic map of the genetic structures surrounding the $bla_{\text{OXA-58}}$ gene in *A.baumannii* strains. The horizontal black arrow indicates the $bla_{\text{OXA-58}}$ gene and the direction of transcription. IS elements are represented by empty rectangular boxes filled with black arrows indicating the transposase gene and the direction of transcription. ST and PFGE types of *A. baumannii* strains showing the corresponding structures are indicated.



References are presented in the general reference list.

CHAPTER 4

Molecular epidemiology of multi-drug resistant *Acinetobacter baumannii* in a tertiary care hospital in Naples, Italy, shows the emergence of a novel epidemic clone.

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Submitted for publication

Molecular epidemiology of multi-drug resistant *Acinetobacter baumannii* in a tertiary care hospital in Naples, Italy, shows the emergence of a novel epidemic clone.

ABSTRACT

The molecular epidemiology of multidrug-resistant *A. 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), tri-locus sequence-based typing (3LST), and multi-locus sequence typing (MLST) of *A. baumannii* isolates from 71 patients identified two distinct genotypes classified as PFGE groups A and B in 14 and 57 patients, respectively. Of these, PFGE group A was assigned to 3LST group 1 and MLST-based ST2, and corresponded to European clone II identified in the same hospital during 2003-2004. PFGE group B was assigned to novel 3LST group 6 and ST78, and was isolated for the first time in May 2006 but became prevalent during 2007. The novel *A. baumannii* clone ST78/B was also isolated in five patients from two additional hospitals in Naples during 2007. The isolates of PFGE groups A and B were resistant to all antimicrobials tested including carbapenems, but were susceptible to colistin. Both isolates of PFGE groups A and B possessed a plasmid-borne carbapenem-hydrolyzing oxacillinase gene $bla_{\text{OXA-58}}$ flanked by IS*Aba2* and IS*Aba3* elements at the 5' and 3' ends, respectively. The selection of the novel *A. baumannii* epidemic clone ST78/B might have been favored by the acquisition of the $bla_{\text{OXA-58}}$ gene.

INTRODUCTION

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 [1,62]. Several hospital outbreaks caused by the selection of multiresistant *A. baumannii* clones have been described in Europe and worldwide [81,1,62,75]. 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 [50,74]. 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 to European clone I, and group 3 to European clone III [23]. 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 [99]. The majority of the outbreaks occurred in Europe were caused by carbapenem-resistant strains that carried the $bla_{\text{OXA-58}}$ gene or a distinct CHDL gene [70,57,67,96,99,90,72,68,2,75,66]. 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 Federico II University hospitals in Naples, Italy, during 2002 [78]. 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 [65]. An increase in the number of cases of *A. baumannii* was observed after two 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, (iii) to analyse the antimicrobial susceptibility 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 intensive care units (ICUs): a neonatal ICU, a coronary ICU, and a cardiac surgery ICU, a general and specialist surgery ICU (namely post-operative ICU, PO-ICU) and a cardio-respiratory ICU (CR-ICU). PO-ICU and CR-ICU are located in a recently renovated area of the hospital, 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 equipments. Patients admitted to PO-ICU are inpatients undergoing major elective surgery, while CR-ICU admits both inpatients requiring intensive care and outpatients from other city or region ICUs. The present study analyzed 71 *A. baumannii* isolates from 71 patients in CR-ICU and PO-ICU wards between May 2006 and December 2007.

MICROBIOLOGICAL SURVEILLANCE AND EPIDEMIOLOGICAL DATA.

Patient microbiological screening at admission to CR-ICU and PO-ICU is routinely performed; further specimens are collected during patients' stay upon clinical judgement. 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-December 2006) was performed retrospectively. Epidemiological data for the 76 *A. baumannii*-positive patients in 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. Microbiological data were analyzed using SPSS v. 11.0 (SPSS Inc., Chicago, IL, USA) by means of Student's t test or Pearson's Chi-squared 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 glycerol 20% v/v. Strains were originally identified as *Acinetobacter baumannii-A. calcoaceticus* complex by using the Vitek 2 automatic system with ID-GNB card for identification of gram-negative bacilli (bioMerieux, Marcy-l'Etoile, France). *A. baumannii* species identification was confirmed by amplification of $bla_{\text{OXA-51-like}}$ gene and PCR amplification and sequence analysis of the 16S-23S rRNA intergenic spacer region [57, 23].

ANTIMICROBIAL SUSCEPTIBILITIES.

MICs were determined by a microdilution method according to Clinical and Laboratory Standards Institute document M7-A6 [67]. Breakpoint values were those recommended from the CLSI [67]. Breakpoints for colistin were those from the British Society for Antimicrobial Chemotherapy (BSAC) [71]. Etest MBL strips (AB BIODISK, Solna, Sweden) were used to evaluate the presence of metallo-beta-lactamase (MBL) activity according to 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 [66].

PULSED-FIELD GEL ELECTROPHORESIS (PFGE) AND DENDROGRAM ANALYSIS.

*Apa*I DNA macrorestriction, PFGE and dendrogram analysis of *A. baumannii* isolates were performed as previously reported. Interpretation of genomic relatedness was performed using Tenover's criteria [73].

IDENTIFICATION OF PCR-BASED SEQUENCE GROUPS AND SEQUENCE-BASED TYPING (ST).

Multiplex PCRs and sequence-based typing were performed as previously described [23]. Assignment of novel alleles and ST types was performed using the bioinformatic tools at the Health Protection Agency web site on *A. baumannii* sequence typing that has been developed and maintained by Drs. J.F. Turton and R. Meyers (http://www.hpabioinformatics.org.uk/AB/home.php).

MULTILOCUS SEQUENCE TYPING (MLST).

MLST analysis was performed using the Institut Pasteur's MLST scheme, publicly available from the MLST web site at http://www.pasteur.fr/mlst. This MLST scheme is based on sequencing of an internal portion of the seven genes 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 and al. [7]. Primer pairs for three other genes (*fusA, pyrG* and *rplB)* are derived from primers initially proposed by Santos and Ochman [119]. Finally, primers for gene *rpoB* were designed previously [120]. 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 IS SEQUENCES.

PCR analysis for carbapenemase-encoding genes in *Acinetobacter* spp. (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SIM}$, *bla*$_{OXA-23-like}$, *bla*$_{OXA-24-like}$, *bla*$_{OXA-51-like}$, *bla*$_{OXA-58}$) was performed as previously described [66]. PCR characterization of the *bla*$_{OXA-58}$-surrounding IS was performed as described [68]. The colinearity between IS elements and *bla*$_{OXA-66}$ or *bla*$_{OXA-90}$ gene was analyzed using primers for IS elements described previously by Poirel and Nordmann [68] and for the *bla*OXA-51-like gene described previously by Turton et al. [79].

PLASMID ANALYSIS.

Plasmid DNA preparations were performed by using the QIAfilter Plasmid purification Maxi kit adapted for low-copy number plasmids (QIAGEN Corporation, Milan, Italy) according to manufacturer's procedure. *Hin*dIII-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 rifampicin, and *Acinetobacter* genomic species 3 strain 4442 [115], susceptible to imipenem while resistant to rifampin, as donor and recipient cells, respectively. Transconjugants were selected on BHI agar plates containing imipenem (16 mg/L) + rifampin (100 mg/L). 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'-GTCACGCCAGTATTAACCAA-3' and 5'-TCGTTTACCCCAAACATAAGC-3', spanning plasmid *oriV* and IS*Aba3*, respectively. DNA sequences 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, USA). DNA sequences were assembled using the program Autoassembler version 1.4 (Applied Biosystems, Foster City, CA, USA) and annotated using the BLAST program [112] 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 ($bla_{\text{OXA-51like}}$ allele 9 corresponding to $bla_{\text{OXA-90}}$), respectively. 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 ST78 are available from the *A. baumannii* MLST web site 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 [65]. During the two subsequent years, only few sporadic *A. baumannii* cases were detected in the hospital. The epidemiology of *A. baumannii* was studied in 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 1760 patients were admitted to the two wards (514 and 1240 to 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 CR-ICU and PO-ICU, respectively, $p<.05$). *A. baumannii* was isolated in 75 CR-ICU patients who showed a mean number of positive specimens of 2.17±1.58. In PO-ICU 26 patients proved to be positive for *A. baumannii,* with a mean number of positive specimens of 2.08±1.35 ($p>.05$). Mean length of stay in the two wards was calculated and proved to be of 10.98±22.32 and 2.22±2.94 days for CR-ICU and PO-ICU, respectively ($p<.05$).

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, 71 available, non-repetitive *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 from PO-ICU (61.5% of patients). One *Acinetobacter* isolate was not included in the study because species identification as *A. baumannii* was not confirmed by molecular methods. Molecular typing by PFGE identified two major PFGE groups, that differed in 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, twelve showed an identical macrorestriction pattern (type A) whereas two showed two- to three-fragment variations and were classified into types A1 and A2, respectively. Fifty-six isolates of PFGE group B showed an identical macrorestriction pattern (type B) whereas one showed a two-fragment variation and was designated as type B1, respectively (Figure 1A and TABLE S1). The epidemic PFGE profile A was identical to that of the epidemic *A. baumannii* strain 2638 isolated in the V. Monaldi hospital during 2004 [65] (Figure 1A). The lower respiratory tract was the most frequent site of isolation (9 of 14 and 45 of 57, for PFGE groups A and B, respectively) ($p>.05$) and was associated with clinical infection as primary diagnosis or infectious

comorbidity in 3 of 10 and 11 of 44 patients for PFGE groups A and B, respectively. Nine *A. baumannii* isolates from blood were assigned to PFGE type B, one to PFGE type A (p>.05), and were always associated with clinical infection. PFGE group B was also isolated from 1 wound swab, the urinary tract of one patient and one catheter tip, while PFGE group A was also isolated from the upper respiratory tract of 1 patient, the central venous catheter and the catheter tip of 2 and 1 patients, respectively. Crude mortality and *A. baumannii*-associated mortality was 64% (9/14) and 21% (3/14), respectively, for patients with isolation of *A. baumannii* of PFGE group A, 75% (43/57) and 25% (14/57), respectively, for patients with isolation of *A. baumannii* of PFGE group B (TABLE S1). Genotype analysis using the multiplex PCRs and tri-locus sequence-based typing approach described by Turton et al [23] assigned all 14 isolates of PFGE group A to previously defined three-locus sequence type (3LST) group 1 (Figure 1B and TABLE S1). The multiplex PCR approach identified a distinct PCR pattern in the other 57 isolates of PFGE group B with the amplification of *bla*$_{\text{OXA-51-like}}$ and *ompA* but not *csuE* alleles in the PCR mix 1 and amplification of *csuE* but not *bla*$_{\text{OXA-51-like}}$ and *ompA* alleles in the PCR mix 2 (Figure 1B). Tri-locus sequence-based typing identified an identical allele profile 7/10/9 at *ompA*/*csuE*/*bla*$_{\text{OXA-51-like}}$ loci in the 57 isolates of PFGE group B that were assigned to a novel 3LST group 6 (TABLE S1). The differences in group-specific PCRs patterns facilitated rapid identification of the *A. baumannii* isolates belonging to different ST groups 1 and 6 into the hospital and were used as preliminary typing approach of the isolates. MLST based on the conserved regions of *cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, *rpoB* housekeeping genes identified ST2 for isolates of PFGE group A, allelic profile 25/3/6/2/28/1/29, which corresponds to a novel ST assigned as ST78 for isolates of PFGE group B, respectively (TABLE S1).

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 two distinct epidemic clones, that were isolated in two consecutive temporal clusters, one clone prevailing over the other. In

fact, clone ST2/A was identified in 10 CR-ICU patients and in 4 PO-ICU patients, while clone 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 CR-ICU ward in May 2006 and predominated in both wards since March 2007 (Figure 2). Five additional MDR *A. baumannii* strains of ST78/B profile were isolated in ICU wards of the Cotugno and A. Cardarelli hospitals in Naples (4 and 1 isolates, respectively) during 2007 (TABLE S1).

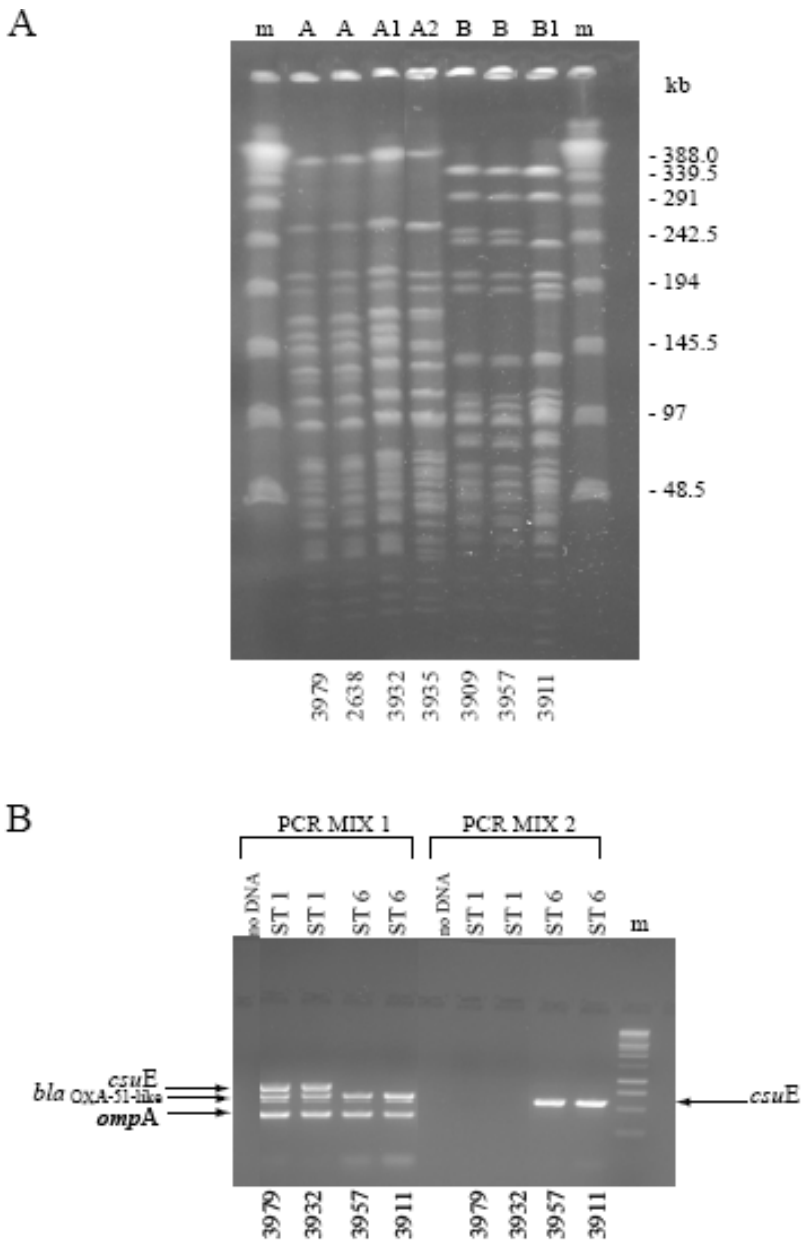

Fig. 1. (A) *Apa*I PFGE profiles of representative *A. baumannii* strains included in the study. Capital letters on the top of the lanes indicate PFGE types identified; m, phage lambda DNA molecular mass markers. Isolate number is shown on the bottom of each lane. Sizes of lambda DNA molecular mass markers are shown on the right-hand side of the panel. (B) Multiplex PCR to selectively amplify *omp*A, *csu*E and $bla_{\text{oxa-51-like}}$ alleles. ST groups identified are indicated on the top of the lanes; m, 1 kb DNA ladder molecular mass markers (Promega, Milan, Italy). Isolate number is shown on the bottom of each lane.

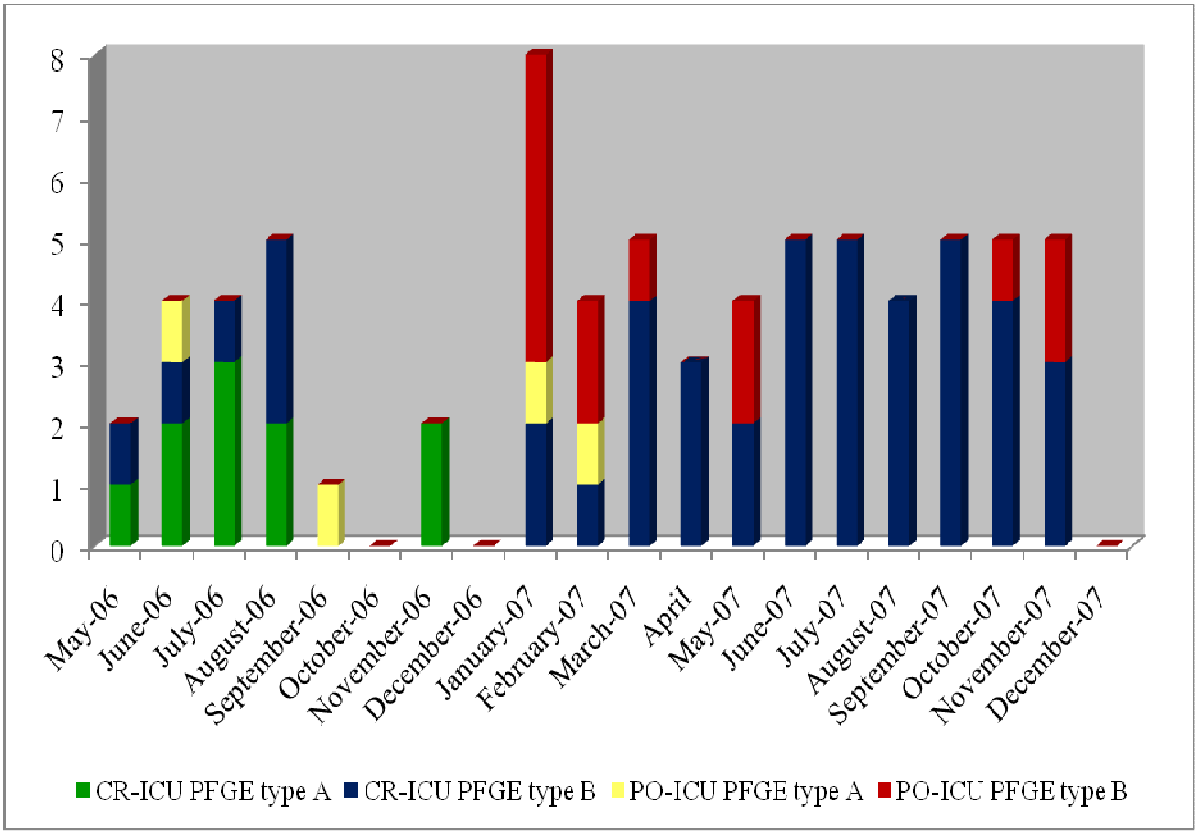

Fig. 2. Molecular epidemiology of *A. baumannii* in the V. Monaldi hospital, Naples, Italy, during 2006–2007. Grey and white bars represent isolates of PFGE group A from cardio-respiratory-ICU (CR-ICU) and post-operative-ICU (PO-ICU), respectively; black and dotted bars represent isolates of PFGE group B from CR-ICU and PO-ICU, respectively.

Antimicrobial susceptibility patterns of *A. baumannii* isolates.

Both *A. baumannii* isolates of PFGE groups A and B from the V. Monaldi hospital showed a multi-drug resistant antibiotype. In particular, they were resistant to ampicillin–sulbactam, piperacillin–tazobactam, broad-spectrum cephalosporins, fluoroquinolones and aminoglycosides, intermediate or resistant to imipenem and rifampicin, intermediate to meropenem, but were susceptible to colistin sulphate. Interestingly, 5 isolates of PFGE group A showed high-level resistance to rifampicin (TABLE 1 and TABLE S1). All four *A. baumannii* isolates of PFGE type B from the Cotugno hospital in Naples showed antimicrobial susceptibility profile identical to isolates of PFGE type B from the V. Monaldi hospital; the single *A. baumannii* isolate of PFGE type B from the A. Cardarelli hospital was susceptible to imipenem (MIC 1.0 mg/liter) and meropenem (MIC 0.5 mg/liter) (TABLE S1). Tests with Etest MBL strips

showed that all carbapenem-resistant isolates of PFGE groups A and B were intermediate or resistant to imipenem (MICs 8–16 mg/liter), but were negative for MBL production (imipenem-EDTA MICs, 4–8 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 isolates of PFGE groups A and B through a microdilution method. These experiments showed that imipenem MICs (16 mg/liter) were inhibited by up to eightfold in the presence of NaCl (2 mg/liter).

TABLE S1. Epidemiological, phenotypic and genotypic features of *A. baumannii* strains included in the study

Strain	Culture date	Ward	Age/ gender	Primary diagnosis	Infectious comorbidity	Outcome	Isolate source	PFGE	Tri-locus ST	MLST	Antibiotic MIC						
								Type	Group	ST	IPM	MEM	SAM	AK	GM	RIF	COL
Monaldi Hospital																	
3978	15/05/2006	CR-ICU	61/M	Respiratory failure	Pneumonia	Exitus	BA	B	6	78	32	16	125	125	32	4	<=0,5
3979	29/05/2006	CR-ICU	85/F	Respiratory failure	Pneumonia	Voluntary discharge	BA	A	1	2	32	8	32	125	8	4	<=0,5
3980	05/06/2006	CR-ICU	67/F	Respiratory failure	Pneumonia*	Exitus	BA	B	6	78	16	8	125	125	32	4	<=0,5
3982	09/06/2006	PO-ICU	81/F	Acute myocardial infarction		Exitus	BA	A	1	2	16	16	32	125	8	2	<=0,5
3993	19/06/2006	CR-ICU	83/F	Pneumonia		Exitus	BA	A1	1	2	16	4	32	125	8	2	<=0,5
3983	28/06/2006	CR-ICU	67/M	Lung cancer	Sepsis	Exitus	BC	A	1	2	16	4	125	4	8	>500	<=0,5
3991	17/07/2006	CR-ICU	79/F	Pneumonia*		Transferred	PS	A	1	2	16	4	32	4	8	4	<=0,5
3994	17/07/2006	CR-ICU	75/M	Aortic dissection		Exitus	BA	B	6	78	8	4	125	125	32	2	<=0,5
3984	17/07/2006	CR-ICU	81/F	Respiratory failure		Discharge	BA	A	1	2	16	8	32	32	8	4	<=0,5
4137	31/07/2006	CR-ICU	67/F	Respiratory failure	Pneumonia	Exitus	BA	A	1	2	8	4	125	125	256	>500	<=0,5
3987	03/08/2006	CR-ICU	47/F	Respiratory failure	Pneumonia	Exitus	BA	B	6	78	16	4	64	125	32	4	<=0,5
3985	14/08/2006	CR-ICU	67/M	Pneumonia		Exitus	BA	B	6	78	16	16	125	16	4	2	1
3992	21/08/2006	CR-ICU	50/F	Respiratory failure		Exitus	BA	A	1	2	32	8	32	125	8	4	<=0,5
3990	23/08/2006	CR-ICU	76/M	Pneumonia		Transferred	CVC	A	1	2	32	16	64	16	4	>500	<=0,5
3989	28/08/2006	CR-ICU	59/M	Esophageal cancer	Pneumonia*	Exitus	BA	B	6	78	16	8	125	125	256	2	1
4138	12/09/2006	PO-ICU	68/M	Lung cancer		Exitus	BA	A	1	2	8	8	32	16	4	>500	<=0,5
3986	06/11/2006	CR-ICU	19/M	Respiratory failure		Exitus	CT	A2	1	2	16	4	32	>250	8	4	<=0,5
3988	06/11/2006	CR-ICU	76/F	Respiratory failure		Discharge	BA	A	1	2	32	4	32	125	8	4	<=0,5
3956	05/01/2007	PO-ICU	26/M	Respiratory failure	Sepsis	Exitus	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*	Exitus	BA	A	1	2	16	16	125	16	64	>500	<=0,5
4156	08/01/2007	PO-ICU	68/M	Larynx cancer		Discharge	BA	B	6	78	8	8	125	>250	8	6	<=0,5
3958	15/01/2007	CR-ICU	81/M	Respiratory failure		Exitus	BA	B	6	78	32	8	125	32	32	16	<=0,5
4157	15/01/2007	PO-ICU	66/M	Colorectal cancer		Voluntary discharge	BA	B	6	78	16	8	125	>250	32	4	<=0,5
3957	18/01/2007	PO-ICU	44/M	Bronchiectasis	Lung abscess	Discharge	BA	B	6	78	16	8	125	32	32	4	<=0,5
3942	24/01/2007	PO-ICU	81/F	Respiratory failure		Voluntary discharge	BA	B	6	78	16	8	125	125	32	16	<=0,5
3997	29/01/2007	CR-ICU	85/F	Respiratory failure		Exitus	BA	B	6	78	16	8	64	16	32	2	<=0,5
4158	03/02/2007	PO-ICU	56/M	Lung cancer		Exitus	CVC	A	1	2	16	16	64	>250	8	>500	<=0,5
3944	12/02/2007	PO-ICU	65/F	Intestinal occlusion		Exitus	BA	B	6	78	16	8	125	>250	32	4	<=0,5
3945	16/02/2007	PO-ICU	39/M	Aortic dissection		Transferred	BA	B	6	78	8	8	64	32	64	4	<=0,5
3960	23/02/2007	CR-ICU	45/M	Pneumocystosis*	Sepsis	Exitus	BC	B	6	78	16	8	125	32	32	2	<=0,5
3998	03/03/2007	CR-ICU	52/F	Respiratory failure	Sepsis	Exitus	BC	B	6	78	16	8	125	>250	32	4	<=0,5
3946	13/03/2007	PO-ICU	75/M	Not specified hemorrhage		Exitus	WS	B	6	78	8	8	125	>250	32	4	<=0,5
3999	19/03/2007	CR-ICU	66/F	Mediastinitis		Exitus	BA	B	6	78	8	4	64	125	32	4	<=0,5
3961	21/03/2007	CR-ICU	77/M	Aortic dissection		Exitus	BA	B	6	78	8	8	125	125	64	2	<=0,5
4000	21/03/2007	CR-ICU	62/M	Respiratory failure		Exitus	BA	B	6	78	16	16	125	125	32	4	<=0,5
3962	10/04/2007	CR-ICU	71/F	Respiratory failure		Exitus	BA	B	6	78	32	4	125	32	32	2	<=0,5
4001	30/04/2007	CR-ICU	78/M	Respiratory failure		Discharge	BA	B	6	78	32	8	125	125	32	4	<=0,5
4002	30/04/2007	CR-ICU	75/F	Pneumonia		Exitus	BA	B	6	78	8	4	125	32	64	4	<=0,5
3963	04/05/2007	CR-ICU	76/F	Respiratory failure		Exitus	BA	B	6	78	16	4	125	32	32	4	<=0,5
3947	08/05/2007	PO-ICU	75/M	Bladder cancer		Discharge	UC	B	6	78	16	4	125	125	32	4	<=0,5
4139	14/05/2007	CR-ICU	57/F	Respiratory failure		Exitus	CT	B	6	78	16	8	64	125	32	4	<=0,5
3948	26/05/2007	PO-ICU	64/M	Colorectal cancer	Sepsis	Exitus	BC	B	6	78	8	8	125	125	32	4	<=0,5

3969	04/06/2007	CR-ICU	74/M	Wegener's granulomatosis		Exitus	BA	B	6	78	8	8	64	16	32	4	<=0,5
4140	05/06/2007	CR-ICU	79/F	Respiratory failure		Exitus	BA	B	6	78	8	8	125	125	32	4	<=0,5
3970	19/06/2007	CR-ICU	52/F	Acute myocardial infarction	Pneumonia	Exitus	BA	B	6	78	16	8	64	16	64	4	<=0,5
4141	21/06/2007	CR-ICU	49/M	Respiratory failure		Transferred	BA	B	6	78	16	4	64	16	32	4	<=0,5
3971	25/06/2007	CR-ICU	68/F	Aortic dissection	Sepsis	Voluntary discharge	BC	B	6	78	32	8	125	125	64	4	<=0,5
4142	28/06/2007	CR-ICU	76/M	Respiratory failure		Exitus	BA	B	6	78	8	4	125	16	32	4	<=0,5
4143	02/07/2007	CR-ICU	54/M	Amyotrophic lateral sclerosis	Pneumonia	Discharge	BA	B	6	78	8	8	125	125	32	4	<=0,5
3972	16/07/2007	CR-ICU	85/M	Respiratory failure	Pneumonia	Transferred	BA	B	6	78	8	8	125	>250	32	4	<=0,5
4145	23/07/2007	CR-ICU	90/M	Acute myocardial infarction		Exitus	BA	B	6	78	16	8	125	125	32	8	<=0,5
4144	24/07/2007	CR-ICU	70/F	Respiratory failure		Exitus	BA	B	6	78	8	8	64	16	32	4	<=0,5
4136	30/07/2007	CR-ICU	84/F	Respiratory failure		Exitus	BA	B	6	78	16	4	125	125	32	4	<=0,5
3966	01/08/2007	CR-ICU	51/M	Renal ptosis	Sepsis	Transferred	BC	B	6	78	32	8	125	125	32	4	<=0,5
4146	02/08/2007	CR-ICU	53/F	Respiratory failure		Exitus	BA	B	6	78	8	4	125	16	32	4	<=0,5
4147	04/08/2007	CR-ICU	70/M	Respiratory failure		Exitus	BA	B	6	78	32	16	125	125	32	4	<=0,5
3967	03/09/2007	CR-ICU	77/F	Respiratory failure		Transferred	BA	B	6	78	8	8	125	125	32	4	<=0,5
4148	03/09/2007	CR-ICU	70/M	Respiratory failure		Exitus	BA	B	6	78	16	8	125	>250	32	2	<=0,5
4149	05/09/2007	CR-ICU	58/F	Respiratory failure	Pneumonia	Exitus	BA	B	6	78	8	4	125	125	32	4	<=0,5
4150	10/09/2007	CR-ICU	76/F	Respiratory failure		Exitus	BA	B	6	78	16	8	125	125	32	4	<=0,5
3974	12/09/2007	CR-ICU	72/M	Respiratory failure	Sepsis	Exitus	BC	B	6	78	8	4	125	16	32	4	<=0,5
4151	01/10/2007	CR-ICU	77/M	Respiratory failure		Exitus	BA	B	6	78	16	16	64	125	32	4	<=0,5
4152	12/10/2007	CR-ICU	63/M	Respiratory failure		Exitus	BA	B	6	78	8	4	125	>250	8	4	<=0,5
3950	12/10/2007	PO-ICU	72/M	Respiratory failure		Exitus	BA	B	6	78	8	4	64	16	32	4	<=0,5

3911	24/10/2007	CR-ICU	74/M	Respiratory failure	Sepsis	Voluntary discharge	BC	B1	6	78	32	8	125	>250	64	4	<=0,5
4153	03/11/2007	CR-ICU	69/F	Respiratory failure		Exitus	BA	B	6	78	8	8	125	125	64	4	<=0,5
3909	05/11/2007	CR-ICU	44/F	Respiratory failure	Pneumonia*	Exitus	BA	B	6	78	32	8	125	2	32	4	<=0,5
3951	14/11/2007	PO-ICU	49/M	Pancreatic cancer		Exitus	BA	B	6	78	16	8	125	2	256	2	<=0,5
4154	19/11/2007	CR-ICU	62/M	Respiratory failure	Pneumonia	Exitus	BAL	B	6	78	16	8	125	2	32	4	<=0,5
3952	24/11/2007	PO-ICU	79/M	Abdominal aortic aneurysm	Sepsis	Exitus	BC	B	6	78	16	8	125	2	32	4	<=0,5
3912	22/10/2007	CR-ICU	45/M	Acute myocardial infarction		Exitus	BA	B	6	78	8	8	125	>250	32	4	<=0,5
Cotugno Hospital																	
3678	07/04/2007	ICU	60/M	Respiratory failure	Pneumonia	Discharge	BA	B	6	78	32	16	125	125	4	4	<=0,5
3679	24/05/2007	ICU	75/M	Cholangiocarcinoma	Sepsis	Discharge	BC	B	6	78	16	8	125	125	32	4	<=0,5
3701	06/06/2007	ICU	60/F	Sepsis		Exitus	BC	B	6	78	16	8	125	125	32	4	<=0,5
3696	03/10/2007	ICU	60/F	Tetanus*		Discharge	CVC	B	6	78	16	8	125	>250	64	4	<=0,5
Cardarelli Hospital																	
3933	04/02/2007	ICU	19/M	Polytrauma	Sepsis	Discharge	BC	B	6	78	1	0,5	125	32	32	4	<=0,5

TABLE 1. Antibiotic susceptibility profiles of *A. baumannii* isolates of PFGE types A and B from the V. Monaldi hospital [a].

	PFGE A (total strains 14)			PFGE B (total strains 57)		
Antibiotic	**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**
Rifampicin	**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 CSLI guidelines. MIC values were expressed as mg/liter.

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* ST/2A and ST/78B isolates, but not from the single carbapenem-susceptible *A. baumannii* strain of PFGE type B isolated in A. Cardarelli hospital. No amplification products were obtained from chromosomal or plasmid DNA of *A. baumannii* ST/2A and ST/78B isolates using primers for *bla*IMP-type, *bla*VIM-type, or *bla*SIMtype 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* isolates of ST/2A and ST/78B profile, respectively, thus

excluding that IS-mediated overexpression of these oxacillinases may account for the resistance to imipenem [79].

Genetic location and characterization of the genetic structures surrounding the *bla*$_{OXA-58}$ gene.

Digestion of plasmid DNA from *A. baumannii* isolates of ST/2A and ST/78B profile with *Hin*dIII 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 hybridization with a *bla*$_{OXA-58}$-specific probe (Fig. 3A). The direct sequence of amplicons generated from plasmid DNA preparation of *A. baumannii* ST/2A and ST/78B isolates using primers spanning the 5' end of *A. baumannii* origin of plasmid replication (*oriV*) and the 3' end of IS*Aba3* element identified two similar fragments of 6095 and 6073-bp, that were designated pABNA1 and pABNA2, respectively. The two amplicons showed identical origin 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 (Figure 3B). Filter-mating experiments demonstrated that resistance to imipenem, along with the *bla*$_{OXA-58}$ gene, was transferred from *A. baumannii* isolate 3957 of PFGE type B, but not from *A. baumannii* isolate 3979 of PFGE type A, to imipenem-susceptible *Acinetobacter* genomic species 3 isolate 4442 at a frequency of 1 x 10^{-6}. Imipenem MICs for transconjugants were similar (16 mg/liter) to those for donor isolates.

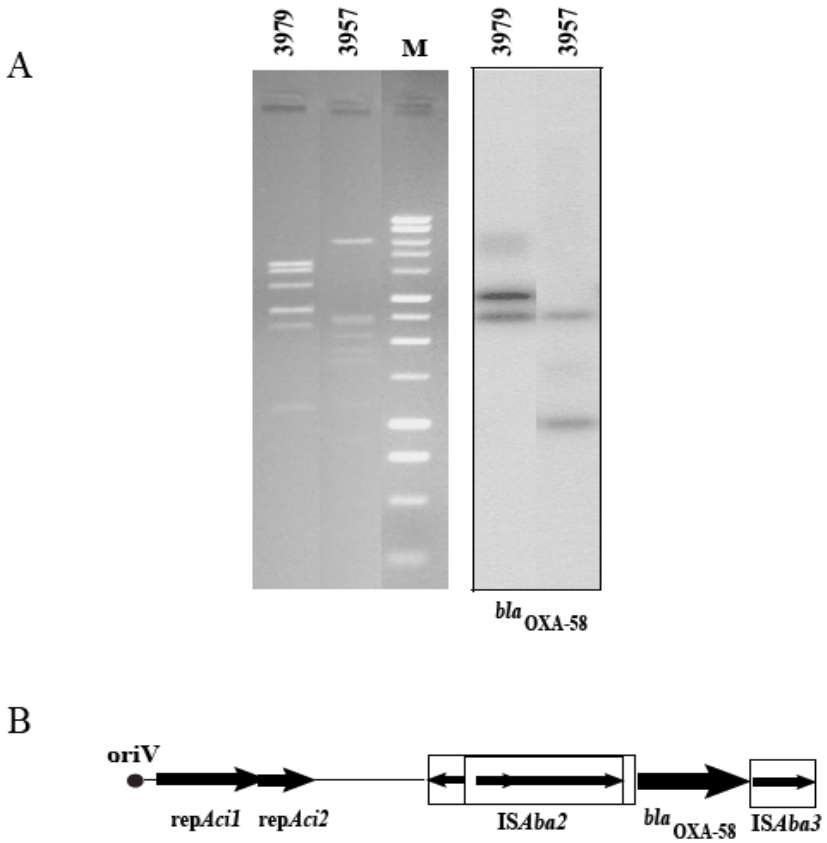

Fig. 3. (A) Plasmid localization of the *bla*$_{OXA-58}$ gene in *A. baumannii* isolates of PFGE groups A and B. Agarose (1%) gel electrophoresis in 1X Tris-acetate-EDTA buffer of *Hin*dIII-digested plasmids from *A. baumannii* isolates, stained with ethidium bromide and visualized under UV light, and Southern blot hybridization with the *bla*$_{OXA-58}$ probe are shown. M is a 1-kb DNA ladder (Promega, Milan, Italy). (B) Schematic map of the genetic structure surrounding the *bla*$_{OXA-58}$ gene in *A. baumannii* isolates of PFGE groups A and B. The genes and their corresponding transcription orientations are indicated by horizontal arrows. IS elements are represented by empty rectangle boxes filled with black arrows indicating the transposase gene and the direction of the transcription. Names of relevant features are reported below or above the map.

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 occurred in two ICUs of the V. Monaldi hospital in Naples. In accordance with previous data [1,62], isolation rate of *A. baumannii* was significantly associated with length of stay in the ward, being higher in CR-ICU than PO-ICU [1,62]. Based on a previous study on an *A. baumannii* outbreak occurred in the same institution between June 2003 and June 2004 [65], 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 profile/genes and near-identity of PFGE profiles indicate that these two sets of isolates each represent a clone. The first epidemic clone showed identical PFGE profile of the *A. baumannii* strains responsible of two epidemics occurred in Naples in the Federico II and V. Monaldi hospitals during 2002 and 2003-2004, respectively [65,71], and was assigned to 3LST group 1 and ST2, that corresponded to the previously characterized European clone II [1,50]. The second epidemic clone, that was first isolated in CR-ICU ward in May 2006 and replaced the previous clone in both wards since March 2007, showed a distinct genotype, that was assigned to a novel 3LST group 6 and ST78, that 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 [1,23,62,92]. 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 [70]. 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 inter-hospital 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 [50,62,65,71]. However, ST78/B strains caused a higher but not statistically significant proportion of bacteremias compared to the other clone, thus suggesting that the novel epidemic clone may possess some inherent properties to develop invasive disease. Several studies demonstrate that *A. baumannii* epidemic strains are selected in the hospital setting because of their multiple antimicrobial resistance [50,70,74,115,118]. In particular, the emergence of carbapenem resistance has been reported during hospital outbreaks of multidrug-resistant *A. baumannii* in Italy and Southern Europe [65,66,70,71,89,92,110,113,115,118]. Accordingly, the two *A. baumannii* clones described in the present study showed a similar antibiotype, characterized by resistance to all classes of antimicrobials including carbapenems, 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* isolate of PFGE type B isolated in 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 [73,113]. 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

[73,89,113] as like as the *A. baumannii* strains responsible for the outbreak occurred in the V. Monaldi hospital during 2003 and 2004 [65,92]. 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 pulsotype [68] and in plasmids isolated in strains from Greece assigned to ST groups 1 and 2 [92]. The above all data 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 [65] 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 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 clone ST2/A to clone ST78/B. 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 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 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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References are presented in the general reference list.

CHAPTER 5

Review article

Carbapenem resistance in *Acinetobacter baumannii*: the molecular epidemic features of an emerging problem in health care facilities

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Carbapenem resistance in *Acinetobacter baumannii*: the molecular epidemic features of an emerging problem in health care facilities

ABSTRACT

Acinetobacter baumannii is an opportunistic gram-negative pathogen with increasing relevance in a variety of nosocomial infections especially among intensive-care-unit (ICU) patients. Carbapenems have been widely used to treat serious multidrug-resistant *A. baumannii* infections; however, incidences of carbapenem-resistant *A. baumannii* are rising in several parts of the world and large and sustained outbreaks caused by such bacteria have been described. Carbapenem-resistant *A. baumannii* epidemics are sustained by clusters of highly similar strains that successfully spread among different cities and countries; their resistance phenotype is mainly due to the acquisition of carbapenem-hydrolyzing class D β-lactamase (CHDL) genes flanked by insertion sequence (IS) elements. Multi-facility outbreaks can be also sustained by inter-hospital transfer of colonized patients. Here, we review the global epidemiology of carbapenem-resistant *A. baumannii*, with the emphasis on the molecular epidemiology and genetic characterization of carbapenem resistance in epidemic strains.

INTRODUCTION

Acinetobacter spp. are glucose-non fermentative gram-negative coccobacilli that have emerged in recent years as a cause of healthcare-associated infections [1,62]. Considered to be commensals of low-grade pathogenicity, i.e. opportunistic microorganisms, *Acinetobacter* were frequently ignored in the 1970s whenever isolated from clinical specimens [1]. The genus *Acinetobacter* currently contains up to 32 described named and unnamed (genomic) species [1]. *Acinetobacter baumannii*, genomic species 3 and 13TU, three of the most clinically relevant species, are genetically and phenotypically very similar to an

environmental species, *A. calcoaceticus*, and are therefore grouped together into the so-called *A. calcoaceticus-A. baumannii* (Acb) complex [1]. Because phenotypic identification of *Acinetobacter* isolates to the species level has proven to be insufficient, several genotypic methods have been developed for genomic species identification, that include amplified 16S rRNA gene restriction analysis (ARDRA), high-resolution fingerprint analysis by amplified fragment length polymorphism (AFLP), or sequence analysis of the 16S-23S rRNA gene spacer region [1,57,59]. However, genotypic methods for species identification are often unavailable in developing countries, where *Acinetobacter* are frequently isolated but identified only at genus level. The species that is most commonly involved in hospital infections is *A. baumannii*, which causes a variety of health-care associated infections, comprising bacteremia, urinary tract infection, surgical-site infection, and nosocomial and ventilator-associated pneumonia, especially in intensive-care-unit (ICU) patients [1,62,65,76,77]. The rates of recovery of *A. baumannii* from natural environments and its incidence in the community are low, while its rate of carriage by hospitalized patients is high and its occurrence in the hospital setting is frequent [1]. *A. baumannii* has simple growth requirements and can survive in dry conditions. This might contribute to the fitness of *A. baumannii* in the hospital environment, which represents the main reservoir of the bacterium [1].

Carbapenem resistance mechanisms in *A. baumannii*

Resistance to antimicrobial agents may be the main advantage of *A. baumannii* in the nosocomial environment. Multidrug-resistant isolates of *A. baumannii* have been reported increasingly during the last decade, probably as a consequence of extensive use of antimicrobial agents in western countries [62,78]. Also, as recently demonstrated by a retrospective, matched cohort study, patients with infection by multidrug-resistant *Acinetobacter* show higher mortality rate and length of hospitalization than patients with infection by susceptible *Acinetobacter* [76].

Mounting evidence indicates that *A. baumannii* possesses a broad range of mechanisms of resistance to all existing antibiotic classes as well as a prodigious capacity to acquire new determinants of resistance [1,62] Genome sequence analysis of six *A. baumannii* clinical strains has shown the presence of a resistance island with a variable composition of resistance genes interspersed with transposons, integrons, and other mobile genetic elements in three of them [79-81]. Also, plasmids carrying resistance genes and/or resistance determinants involved in horizontal gene transfer have been described in several *A. baumannii* strains [63,66,68,82-86].

The broad-spectrum β-lactam antibiotics, carbapenems, were introduced by 1985 and have been for years the most important agents for the treatment of infections caused by multidrug-resistant *A. baumannii*. Carbapenem resistance in *Acinetobacter* is now observed increasingly worldwide, and constitutes a sentinel event for emerging antimicrobial resistance [62,82]. Carbapenem-resistant isolates of *A. baumannii* are usually resistant to all classes of antimicrobials, and show intermediate resistance to rifampin, while usually retaining susceptibility to tigecycline and colistin [62,82,87]. Resistance against carbapenems is, in itself, considered sufficient to define an isolate of *A. baumannii* as highly resistant [82]. The resistance of *A. baumannii* to carbapenems can be mediated by one of the resistance mechanisms that are known to occur in bacteria, including enzymatic inactivation, active efflux of drugs, and modification of target sites (Table 1). The production of carbapenem-hydrolizing beta-lactamases is the most common mechanism responsible for carbapenem resistance in *A. baumannii*. Several carbapenem-hydrolyzing β-lactamases have been identified so far in *A. baumannii*. These include metallo-β-lactamases (VIM-, IMP- and SIM-types), which have been sporadically reported in some parts of the world and have been associated with class 1 integrons [62,77,82]. Nevertheless, the most widespread carbapenemases in *A. baumannii* are class D β-lactamases. Three main acquired carbapenem-hydrolysing class D oxacillinase (CHDL) gene clusters have been identified either in the chromosome or in plasmids of *A. baumannii*

strains, represented by the *bla*$_{\text{OXA-23-}}$, *bla*$_{\text{OXA-24/40-}}$, and *bla*$_{\text{OXA-58-like}}$ genes [82]. Different insertion sequence (IS) elements at the 5' and/or the 3' end of *bla*$_{\text{OXA-23-}}$, and *bla*$_{\text{OXA-58-like}}$ genes, such as IS*Aba1*, IS*Aba2*, IS*Aba3*, or IS*18*, have been demonstrated to regulate their expression [66,68,78,83,84]. Also, it has been recently demonstrated that the IS*Aba1* element is capable of transposition in *E. coli* and of mobilizing an antibiotic resistance gene [85]. In addition to these CHDL genes, the chromosomal *bla*$_{\text{OXA-51-like}}$ gene, intrinsic to *A. baumannii* species, has been demonstrated to confer carbapenem resistance when an IS*Aba1* element is inserted upstream of the gene [86]. Reduced susceptibility to carbapenems has also been associated with the modification of penicillin-binding proteins and porins or with upregulation of the AdeABC efflux system, and it has been suggested that the interplay of different mechanisms might result in high-level carbapenem resistance in *A. baumannii* (Table 1) [88-90].

Table 1.Carbapenem mechanisms in *A. baumannii*.

Mechanism or responsible structure	Note	References
β-lactam hydrolysis		
IMP-1, -2, -4, -5, -6, -11 VIM-2, SIM-1	Class B metallo beta-lactamases. Class 1 integron-associated genes.	2,12
OXA-23 cluster	Class D beta-lactamases. Chromosomal or plasmid genes flanked by IS elements.	2,12,13,17
OXA-24/40 cluster	Class D beta-lactamases. Chromosomal or plasmid genes.	2,12
OXA-58 cluster	Class D beta-lactamases. Plasmid or chromosomal genes flanked by IS elements.	12-16
OXA-51cluster	Chromosomal class D beta-lactamase intrinsic to *A. baumannii*. Confers carbapenem resistances if IS elements are inserted upstream of the gene	2,19
Changes in outer-membrane proteins (OMPs)		
CarO	26 kDa OMP implicated in drug influx	21
33 to 36-kDa OMP OprD-like OMP	Other OMPs associated with carbapenem resistance	2,12
Target alteration		
Altered penicillin-binding proteins	Reduced PBP-2 expression	22

Global epidemiology of carbapenem-resistant *Acinetobacter baumannii*

Carbapenem resistance in *A. baumannii* is now an emerging issue worldwide [62]. Surveillance studies indicate that the percentage of carbapenem-resistant isolates gradually increased over the last ten years in Europe, North America, and Latin America [62]. Numerous outbreaks of carbapenem-resistant *A. baumannii* were reported from hospitals in Northern Europe (Spain, Portugal, France, the United Kingdom (UK), the Netherlands, Czech Republic, Poland) [1,26,62,91-95], Southern Europe and the Middle East (Bulgaria, Greece, Italy, Turkey, Lebanon, Israel, Iran, Iraq and United Arab Emirates) [62,63,65,68,77,78,80,82,84,96-101], North America and Latin America (Argentina, Brazil, Chile and Colombia) [62,102,103], Tunisia and South Africa [104,105], China, Taiwan, Singapore, Hong Kong, Japan, South Korea [62,106,107], and Australia [108] and from areas as remote as French Polynesia [109]. In the majority of cases, one or two epidemic strains were detected in a given hospital. Transmission of such strains was observed between hospitals in the same city and also on a national scale [1,26,62,65,82,91-93,95,96,104,108,110-112] and a direct epidemiological link was established in several cases [26,65,94,95,104,108,110-112]. The inter-hospital transfer of colonised patients was demonstrated during multi-facility outbreaks that occurred in the Netherlands [26], Italy [65], South Africa [104], and Tunisia [105]. The international transfer of patients colonised by carbapenem-resistant *A. baumannii* was also reported [94,95,108]. More recently, several cases of United Kingdom and US military and nonmilitary personnel returning from operations in Iraq and Afghanistan and harbouring infections caused by carbapenem-resistant *A. baumannii* were reported [110-112] (Figure 1).

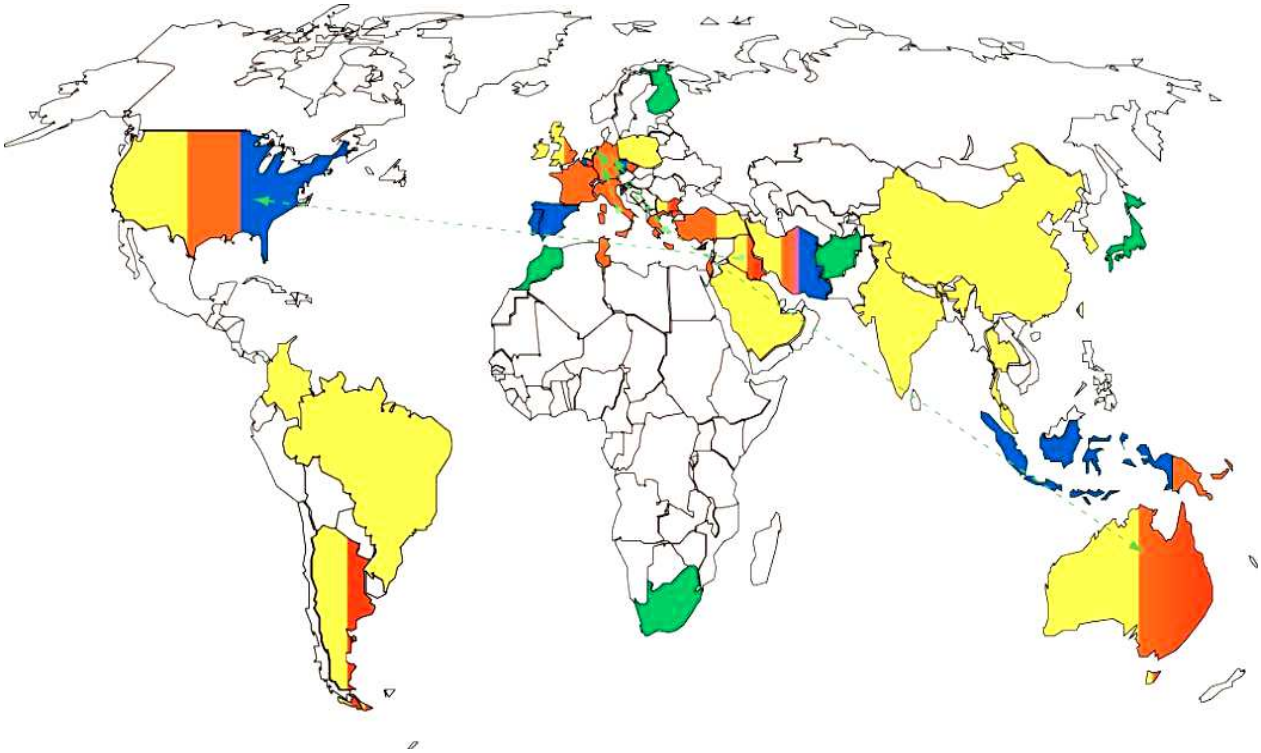
Outbreaks caused by carbapenem-resistant *A. baumannii* have also been observed in developing countries such as Morocco, Thailand, India, and Indonesia [113,107]. Furthermore, infections caused by *Acinetobacter* spp. without specifying whether they are caused by

carbapenem-resistant strains have been reported in Africa (Lagos, Nigeria) and several Asian countries including Nepal [114-116].

Molecular epidemiology of carbapenem-resistant *Acinetobacter baumannii*

Genotypic characterization of carbapenem-resistant *A. baumannii* strains showed the occurrence of *bla*$_{\text{OXA-23-}}$, *bla*$_{\text{OXA-24/40-}}$, or *bla*$_{\text{OXA-58-like}}$ genes in multiple isolates from the same hospital or among different hospitals worldwide [62,82,68,99,100,109,118]. *bla*$_{\text{OXA-23}}$ was mostly detected in isolates from Asian countries [107], but was also reported in South America [102,103] and Europe [82,84,97,117]; *bla*$_{\text{OXA-58}}$ was frequently found in Europe [65,77,80,63,96,99,66,96-101,117]. *bla*$_{\text{OXA-24/40}}$ was mostly found in the Iberian peninsula and Asia, but also detected in Iran, Belgium, Czech Republic and the United States of America (USA) [62,92,93,107,110,117,118] (Figure 1). Molecular epidemiology of *A. baumannii* strains responsible for outbreaks that occurred in several European hospitals revealed clusters of highly similar strains, which were defined as European clones I and II [1,62] and corresponded to sequence type (ST) groups 2 and 1, respectively, identified by sequence-based typing [23]. A recent study on a collection of 96 carbapenem-resistant *A. baumannii* strains collected in 17 European countries assigned 85% of them to sequence type (ST) groups 1 and 2 by multiple PCRs [117]. The prevalence of carbapenem-resistant epidemic *A. baumannii* strains belonging to ST group 1 was also demonstrated in Italy and Greece [96,99] along with the spread of a prevalent clone isolated with identical pulsed field gel electrophoresis (PFGE) profiles in two hospitals in Naples, Italy, and in three hospitals in three distinct Greek cities [99]. The circulation of distinct carbapenem-resistant *A. baumannii* genotypes belonging to ST group 2 in Greece and in Lebanon, and to two novel ST groups 4 and 5 in different Greek and Turkish cities, was also shown in the same study [99]. The *bla*$_{\text{OXA-58}}$ gene flanked by IS elements was present in all carbapenem-resistant genotypes analyzed from hospitals in Greece, Italy, Lebanon, and Turkey [77,66,99] (Figure 1). Of note, each of the IS

elements flanking the 5' end of *bla*$_{OXA-58}$ occurred in strains of distinct ST groups and PFGE profiles isolated in the same geographic region. Thus, IS*Aba2* element was detected in Greece and Italy, IS*18* in Lebanon and Turkey, and IS*Aba1* in Turkey and Italy, suggesting that they might have been acquired through horizontal gene transfer [99]. In further support of this hypothesis, plasmid-borne *bla*$_{OXA-58}$ has been found in the majority of carbapenem-resistant *A. baumannii* strains isolated in Europe [65,77,80,68,63,66,99]. The spread of carbapenem-resistant *A. baumannii* carrying the *bla*$_{OXA-58}$ gene might had also been contributed by international transfer of colonised patients, as recently demonstrated from Greece to Belgium [94], Greece to Australia [108], and Iraq to USA military services [110] (Figure 1).



Geographic distribution and genetic characterization of carbapenem-resistant *A. baumannii*. Countries reporting carbapenem-resistant *A. baumannii* outbreaks producing OXA-23-, OXA-24/40-, and/or OXA-58-type enzymes are indicated by yellow, blue, and red colours, respectively. Countries reporting carbapenem-resistant *A. baumannii* outbreaks in which the OXA-type enzyme has not been identified are indicated by green colour. Green arrows indicate hospital transfer of colonized/infected patients by carbapenem-resistant *A. baumannii* between different countries.

CONCLUSIONS

Outbreaks of carbapenem-resistant *A. baumannii* are increasingly reported in several parts of the world that also include developing countries. They are sustained by clusters of highly similar strains that successfully spread among different cities and countries and are selected because of the acquisition of CHDLs genes flanked by IS elements. Multi-facility *A. baumannii* outbreaks can be also sustained by inter-hospital transfer of colonized patients. This emphasizes the need to adopt surveillance and infection control programmes to prevent colonisation and infection by multidrug-resistant *A. baumannii* in the hospital setting. These programmes would include the study of global epidemiology of multidrug-resistant *A. baumannii* using molecular typing of bacterial isolates and characterization of antibiotic resistance in order to control the spread of *A. baumannii* infections over a wide geographic region.

Acknowledgments
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Restriction placed on the number of references that could be cited in this review mean that, in many cases, either a single paper or a review is cited. We apologize to those authors whose work has not been cited.

References are presented in the general reference list.

ADDENDUM

Nucleotide Plasmid Sequences pABNA1and pABNA2 deposited in the Genbank Nucleotide Database

GenBank: GQ338082.1

Acinetobacter baumannii plasmid pABNA1 RepAci1 (repAci1) and RepAci2 (repAci2) genes, complete cds; insertion sequence ISAba3 tnpA gene, partial cds; insertion sequence ISAba2 insA (insA) and insB (insB) genes, complete cds; insertion sequence ISAba3 tnpA gene...

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05-AUG-2009
DEFINITION  Acinetobacter baumannii plasmid pABNA1 RepAci1 (repAci1)
and RepAci2 (repAci2) genes, complete cds; insertion sequence ISAba3
tnpA gene, partial cds; insertion sequence ISAba2 insA (insA) and insB
(insB) genes, complete cds; insertion sequence ISAba3 tnpA gene,
partial cds; OXA-58 (blaOXA-58) gene, complete cds; and insertion
sequence ISAba3 tnpA gene, partial cds.
ACCESSION   GQ338082
VERSION     GQ338082.1  GI:254972045
KEYWORDS    .
SOURCE      Acinetobacter baumannii
  ORGANISM  Acinetobacter baumannii
            Bacteria; Proteobacteria; Gammaproteobacteria;
Pseudomonadales;
            Moraxellaceae; Acinetobacter.
REFERENCE   1  (bases 1 to 6095)
  AUTHORS   Zarrilli,R. and Giannouli,M.
  TITLE     Molecular epidemiology of multi-drug resistant
Acinetobacter
            baumannii in a tertiary care Hospital in Naples, Italy,
shows the
            emergence of a novel epidemic clone
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 6095)
  AUTHORS   Zarrilli,R. and Giannouli,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-JUN-2009) Preventive Medical Sciences,
University of
            Naples 'Federico II', Via S. Pansini nr. 5, Napoli 80131,
Italy
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GenBank: GQ338083.1

Acinetobacter baumannii plasmid pABNA2 RepAci1 (repAci1) and RepAci2 (repAci2) genes, complete cds; insertion sequence ISAba3 tnpA gene, partial cds; insertion sequence ISAba2 insA (insA) and insB (insB) genes, complete cds; insertion sequence ISAba3 tnpA gene...

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05-AUG-2009
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and RepAci2 (repAci2) genes, complete cds; insertion sequence ISAba3
tnpA gene, partial cds; insertion sequence ISAba2 insA (insA) and insB
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REFERENCE   1  (bases 1 to 6073)
  AUTHORS   Zarrilli,R. and Giannouli,M.
  TITLE     Molecular epidemiology of multi-drug resistant
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shows the
            emergence of a novel epidemic clone
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 6073)
  AUTHORS   Zarrilli,R. and Giannouli,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-JUN-2009) Preventive Medical Sciences,
University of
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