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Epidemiological characterization and distribution of carbapenem-resistant *Acinetobacter baumannii* clinical isolates in Italy

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Abstract

This study was aimed at tracing the molecular characteristics of carbapenem-resistant *Acinetobacter baumannii* (CRAB) clinical isolates in Italy with both pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Two hundred and two CRAB isolates were collected during 2004–2009, in two different surveillance periods, from 22 Italian hospitals that were representative for both distribution and infection. PFGE was performed, and the MLST scheme used was based on the gene sequence as published on the MLST Pasteur website http://www.pasteur.fr/mlst. Representatives of the major European clones I (RUH 875) and II (RUH 134) were used as controls. The two groups of isolates were characterized for their carbapenem resistance genes: 154 of 202 carried bla_{OXA-58} alone, 21 of 202 also carried bla_{OXA-23}, and 27 of 202 carried bla_{OXA-23} alone. No isolates were positive for bla_{OXA-24}. Genotype analysis of all isolates identified four distinct patterns by PFGE, which correlated with four distinct sequence types (STs) by MLST. The distribution of these four clusters in Italy confirmed the propensity of *A. baumannii* for nosocomial cross-transmission in a vast geographical area. We observed that clones A and B had similarities with European clone II and I respectively. By MLST, clone A was ST2, like European clone II, and clone B was ST1, like European clone I. PFGE and MLST showed the same discriminatory power and reproducibility. In addition, the two methods were concordant in defining CRAB Italian clones and in correlating them with the two pan-European clones.

Keywords: *Acinetobacter baumannii*, carbapenemases, MLST, PFGE, tigecycline
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Introduction

Acinetobacter baumannii is an important opportunistic pathogen that is rapidly evolving towards multidrug resistance (MDR) and is involved in various nosocomial infections, especially in intensive-care units (ICUs), with increasing frequency [1–3]. This microorganism is usually associated with severe hospital infections [4], which often need the use of carbapenems as a last-resort treatment. However, reduced susceptibility or resistance to carbapenems is increasingly being observed among *A. baumannii* clinical isolates [5–9].

Numerous nosocomial outbreaks have already been reported in Italy, with many reports emphasizing the association of antibiotic resistance and outbreak strains [10,11]. Extensive genotypic characterization has shown that clusters of highly similar *A. baumannii* strains occur, representative of distinct clonal lineages. Of these, the so-called European clones I, II and III are widely spread across Europe, and include strains that are usually multidrug resistant and associated with outbreaks of hospital infections [12–14]. Changes in the prevalence of carbapenem resistance genes of the OXA type in *A. baumannii* circulating among the urban area of Rome have recently been documented, with bla_{OXA-23} gradually replacing bla_{OXA-58} [15]. It has been suggested that this phenomenon could be related, at least in part, to the higher carbapenemase

activity of bla_{OXA-23} than of bla_{OXA-58}, which results in a higher imipenem MIC and in a clear resistance phenotype for stains carrying this determinant. However, extensive data on the prevalence of these genes throughout Italy are still lacking.

To investigate the molecular epidemiology of *A. baumannii*, a variety of typing systems have been developed, including ribotyping, genome analysis with selective amplified fragment length polymorphisms, randomly amplified polymorphic DNA analysis, infrequent-restriction-site PCR, and pulsed-field gel electrophoresis (PFGE) [16,17]. PFGE restriction analysis of chromosomal bacterial DNA has been used with excellent results in epidemiological studies of numerous *A. baumannii* outbreaks, and is currently regarded as the reference standard for epidemiological typing [18]. Another possible approach involves the use of multilocus sequence typing (MLST), a highly discriminative method based on the sequence comparison of internal fragments of housekeeping genes, which has been applied to a variety of bacterial pathogens, and, most recently, also to *A. baumannii* [19,20].

This national study was carried out with the aim of: (i) obtaining epidemiological typing data for Italian carbapenem-resistant *Acinetobacter baumannii* (CRAB) clinical isolates collected during two different periods, using both PFGE and MLST; (ii) establishing clonal relationships with European clones; and (iii) assessing the actual prevalence of carbapenem resistance genes in the investigated isolates.

Materials and Methods

Bacterial strains

Two hundred and two CRAB non-replicate isolates, responsible for severe infections, were collected from 22 hospitals distributed in 14 towns in Italy during two different surveillance periods (2004–2005 and 2008–2009). During the first period, 120 isolates were obtained from 120 patients, and in the second period, 190 isolates were obtained from 136 patients; however, after elimination of duplicate strains, the total was 82. Clinical data collected included age, sex, number of days in hospital prior to isolation, specimen types, wards, and type of infection. Isolates were mainly obtained from lower respiratory tract infections and documented bloodstream infections; only a limited number of isolates came from complicated skin and skin structure infections, skin and skin structure infections, intra-abdominal infections, and urinary tract infections. Isolates were collected by standard methods, isolated in pure culture on MacConkey agar plates, and identified with the Vitek 2 system (bioMerieux, Marcy l'Etoile, France). Identification at the species level was centrally reconfirmed with the API 20NE system (bioMerieux).

Molecular typing: PFGE and MLST

Genotyping of all organisms identified as being *A. baumannii* was performed with *Apa*I digestion followed by PFGE, using a method described previously [21]. Genomic DNA was prepared in agarose plugs, and DNA restriction was carried out at 30°C for 16 h. PFGE was performed in a CHEF DRII system (Bio-Rad, Hercules, CA, USA), with pulses ranging from 0.5 to 15 s at a voltage of 6 V/cm at 14°C for 20 h. Lambda 48.5-kb concatamers (New England BioLabs, Beverly, MA, USA) were used as molecular size markers. Isolates showing three or fewer band differences were regarded as a single PFGE type, according to the criteria described previously by Tenover *et al.* [22]. MLST was performed on 21 isolates, selected on the basis of the different PFGE types and subtypes: in particular, five isolates belonging to PFGE type A and two isolates belonging to PFGE type B from both study periods, three type C isolates, and four type D isolates. Representatives of major European clones I (RUH 875) and II (RUH 134) were used as controls.

Genotyping by MLST was performed with primers and conditions described on the Pasteur website http://www.pasteur.fr/mlst, with the exception of *rpoB* amplification, which was performed with primers *rpoB*-f (5′-GGCGAAATAGTAG AACCAC-3′) and *rpoB*-r (5′-AGCTTCGAAGTTGTAACC-3′), designed on the basis of a published sequence (CU459141). All PCR amplifications were carried out under the following conditions: 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 2 min, preceded by a 5-min denaturation at 94°C and followed by a 10-min extension at 72°C. PCR products were directly purified from the reaction mixture with the QIAquick PCR purification kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. Purified products were sequenced by an external facility (BioMolecular Research), and results were analysed with the VectorNTI software package and compared with the *A. baumannii* database on the MLST website http://www.pasteur.fr/mlst.

PCR of carbapenem resistance genes

PCR assays were carried out by using previously published primers for amplification of genes encoding carbapenemases (bla_{IMP}, bla_{VIM}, $bla_{OXA-23-like}$, $bla_{OXA-24-like}$, $bla_{OXA-51-like}$, and bla_{OXA-58}) [23,24]. The $bla_{OXA51-like}$ gene was also evaluated as an identification marker [25].

The PCR assays were performed directly on colonies, and the obtained products were purified and sequenced (BioMolecular Research).

Antimicrobial agents and MIC determination

MIC determinations were performed by microdilution in cation-adjusted Mueller–Hinton broth, in accordance with

the 2010 CLSI guidelines [26]. The following antibiotics were tested: meropenem, imipenem, piperacillin, piperacillin–tazobactam, aztreonam, ceftazidime, cefepime, ciprofloxacin, amikacin, gentamicin, colistin, and tigecycline. MIC values were interpreted with category designations according to CLSI criteria. No interpretive criteria have been approved for tigecycline when testing *Acinetobacter* spp. [27]. *Escherichia coli* ATCC 25922 was used as control.

Results

Isolates, wards, and clinical specimens

Table 1 shows the characteristics of CRAB isolates collected during the two study periods. In 2004–2005, 120 isolates (51% from males and 49% from females) were included, with patient ages ranging from 1 to 94 years (mean age, 68 years). During 2008–2009, a further 82 isolates were collected (70% from males and 30% from females), with patient ages ranging from 7 to 90 years (mean age, 62 years). Significant changes in the presence of CRAB isolates were observed in ICU patients between the two periods, with an incidence of 74% in the first period, and a reduction to 40% in the second period. In contrast, in medicine and pneumology wards, increases of 25% and 7%, respectively, were observed. This discrepancy is attributable to the duplicates being eliminated from the second period.

In both periods, approximately 5% and 6% of the isolates came from surgery units and from transplantation units, respectively. All isolates were collected from various types of nosocomial infection, such as respiratory tract infections (32% in 2004 and 35% in 2008), bloodstream infections, and, less frequently, urinary tract infections.

Respiratory secretions, urine and blood were the most common clinical specimens collected, without significant differences between the two periods.

TABLE 1. Clinical characteristics of patients (*N* = 202) with carbapenem-resistant *Acinetobacter baumannii* infections

Patient characteristics	No. (%) of patients, 2004–2005	No. (%) of patients, 2008–2009
Sex		
Male	61 (51)	57 (70)
Female	59 (49)	25 (30)
Age (years), range (mean)	1–94 (68)	7–90 (62)
Admission: ICU	89 (74)	33 (40)
Non-ICU wards		
Medicine	16 (14)	31 (39)
Surgery	8 (6)	4 (5)
Transplantation	6 (5)	7 (8)
Pneumology	1 (1)	7 (8)
No. of days in hospital prior to isolation, range (mean)	3–60 (32)	5–57 (31)
Severe infections		
Lower respiratory tract	39 (32)	29 (35)
Blood	25 (21)	14 (17)
Urinary tract	9 (7)	7 (8)
Other	47 (40)	32 (40)
Specimens		
Lung	53 (44)	33 (41)
Blood culture	17 (14)	15 (18)
Urine	10 (9)	9 (16)
Other	40 (33)	25 (31)
Total	**120**	**82**

ICU, intensive-care unit.

Molecular typing: PFGE and MLST

To trace the molecular characteristics of the CRAB isolates, they were all genotyped by PFGE and MLST. PFGE analysis was able to classify the 120 isolates of 2004–2005 into three PFGE clones, named A, B, and C, three main clone A subtypes, A_1, A_2, and A_3, and two main clone C subtypes, C_1 and C_2. The three PFGE clones coincided with sequence types (STs) found by MLST. In fact, the three PFGE clones belonged to three different STs (clone A was ST2, clone B was ST1, and clone C was ST20). Ninety-two per cent of isolates belonged to clone A (ST2), as shown in Table 2a.

PFGE clone B (ST1) and clone C (ST20) were found in two hospitals of southern Italy, Bari and Palermo, respectively. Six strains belonging to clone C were distributed in two wards of a hospital and, in particular, one of these (C1) was isolated in a medical unit and five (C1 and C2) in an ICU.

As regards the strains collected in 2008–2009, clone A (ST2) represented 81% of CRAB isolates, and clone B (ST1), previously isolated only in Bari, persisted in this area and was also found in two hospitals in central (Rome S. Pertini) and northern (Novara) Italy (Table 2b).

Interestingly, a new PFGE clone, named clone D and belonging to ST78, was detected in two hospitals in southern Italy (Catania Policlinico; Naples Federico II) and in a hospital in northern Italy (Novara), and represented 13% of the isolates. The comparison among Italian and European clones I and II highlighted the fact that PFGE clone A (ST2) was related to European clone II, and PFGE clone B (ST1) was related to European clone I (Fig. 1).

Antimicrobial agents, MIC determinations, and carbapenem resistance genes

The results of the *in vitro* susceptibility testing in the two study periods, expressed as percentage of resistance, are presented in Table 3. All isolates were resistant to meropenem in both periods, whereas the activity of imipenem showed some differences: 90.8% in 2004–2005 and 100% in 2008–2009. Most *A. baumannii* isolates were multidrug resistant, showing resistance to: β-lactams (piperacillin, piperacillin–tazobactam, aztreonam, and ceftazidime), fluoroquinolones (ciprofloxacin), and aminoglycosides (gentamicin).

TABLE 2. Genotypic and epidemiological analysis of strains isolated during: (a) 2004–2005; and (b) 2008–2009

No. of strains	PFGE type	PFGE subtype	ST	Hospitals	OXA content		
					58	58 + 23	
(a)							
110	A	A1, A2, A3	2	Bari, Naples Federico II, Naples Cardarelli, Naples Tufano, Rome S. Camillo, Avellino, Pavia S. Matteo, Pavia Maugeri, Reggio Calabria, Florence, Turin, Foggia and Varese	110	0	
4	B		1	Bari	4	0	
6	C	C1, C2	20	Palermo	0	6	
120					**114**	**6**	
Clone II (RUH 134)	A	–	2				
Clone I (RUH 875)	B	–	1				
(b)							
					OXA content		
					58	**58 + 23**	**23**
66	**A**	A1, A2, A3	2	Bari, Naples Federico II, Catania OVE, Rome Gemelli, Rome Umberto I, Palermo Chiarini, Milan and Pavia S. Matteo	27	12	27
5	B		1	Novara, Rome S. Pertini and Bari	2	3	
11	D		78	Naples Federico II, Novara and Catania Policlinico	11	0	
82					**40**	**15**	**27**

PFGE, pulsed-field gel electrophoresis; ST, sequence type.

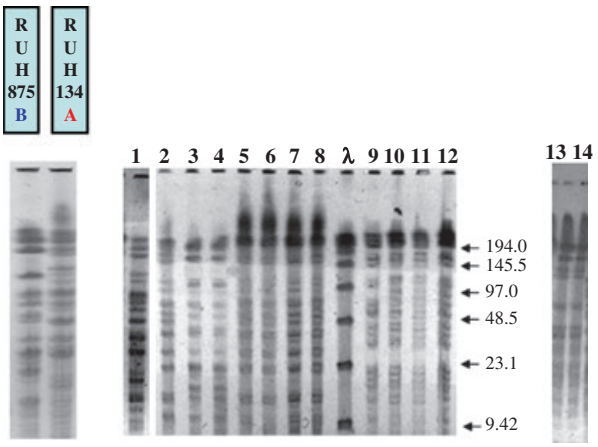

FIG. 1. PEGE *Apa* I profiles of representative *A. baumannii* strains including European clones I (RUH 875) and II (RUH 134). λ: molecular size marker in kilobases. Lane 1 clone A_2/ST 2, lane 2 clone A_3/ST2, Lanes 3–4-clone B/ST 1, lanes 5–6–7–8-clone A_1, lane 9–10 clone C_2/ST20, lanes 11–12 clone C_1/ST 20 and lane 13–14 clone D/ST 78.

All of the 120 isolates obtained in the first period were resistant to piperacillin and ceftazidime, and 98% were resistant to aztreonam and ciprofloxacin. Only one isolates was resistant to colistin, and the results concerning the activity of tigecycline showed moderate activity. Only eight of 120 multidrug-resistant isolates showed tigecycline MIC values between 4 and 8 mg/L.

Increased levels of resistance to all antibiotics tested were observed during the second period; 23 of 82 isolates had MICs of 4 and 8 mg/L for tigecycline, and all isolates were susceptible only to colistin.

All isolates were characterized for their carbapenamase genes: they were all metallo-β-lactamase-negative, but positive for carbapenem-hydrolysing class D β-lactamase genes. All of the clones found in the first period carried $bla_{OXA\text{-}58}$, and only one of them, clone C, carried both $bla_{OXA\text{-}58}$ and $bla_{OXA\text{-}23}$. In the second period, $bla_{OXA\text{-}58}$ was detected in 40 of 82 isolates belonging to clones A, B, and D. Fifteen of 82 isolates belonging to clone A and B carried both $bla_{OXA\text{-}58}$ and $bla_{OXA\text{-}23}$. Finally, 27 of 82 isolates collected at S. Matteo hospital in Pavia carried $bla_{OXA\text{-}23}$, and all isolates belonged to clone A; therefore, this clone was responsible for an outbreak in this hospital. All isolates were positive for $bla_{OXA\text{-}51}$, the carbapenemase gene that is intrinsic to this species.

Discussion

A. baumannii is one of the most widespread microorganisms responsible for global outbreaks, displaying ever increasing rates of resistance [28]. There are reports of multidrug-resistant *A. baumannii* strains from Europe and North America, Russia, Brazil, China, and Tahiti [29–33]. Multidrug-resistant strains often spread, causing outbreaks throughout entire cities, countries, and continents [34]. Furthermore, the importation of multidrug-resistant strains from areas with high rates of antimicrobial resistance to areas with historically low rates, such as from Spain to Norway, has been demonstrated. More recently, several cases of UK and US

TABLE 3. MIC distribution and antibiotic susceptibility of *Acinetobacter baumannii* (*N* = 202).

	MIC (mg/l)														2004	2008
Antibiotics	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	(%R)	
Imipenem								5	6	22* / 42**	74 / 9	11 / 10	2 / 12	9	90,8	100
Meropenem										12 / 8	8 / 8	52 / 14	32 / 24	16 / 28	100	100
Piperacillin														120 / 82	100	100
Piperacillin/ Tazobactam					3			3		48	2 / 3	3	2 / 65	68 / 5	58. 3	85.4
Aztreonam								2		2		116 / 12	51	19	98. 3	100.0
Ceftazidime							3	3		2	38	120 / 3	22	11	100.0	90.2
Cefepime							3	4 / 3	30	2 / 5	72 / 32	12 / 3	33	3	70.0	86.6
Ciprofloxacin					2			2 / 47	116 / 35						98. 3	100.0
Amikacin		2					8	34 / 10	2		19 / 8	65 / 16	34	4	54.2	75.6
Gentamicin			2				3	25	1	69 / 40	25 / 27	10			78. 3	93.9
Colistin			15	19 / 60	19	34 / 22	32			1					0.8	0
Tigecycline	10			2 / 5	6	27 / 10	77 / 34	6 / 22	2 / 1						-	-

*2004–2005, **2008–2009.

military and non-military personnel returning from operations in Iraq and Afghanistan, and harbouring infections caused by CRAB, have been reported [35,36].

CRAB is now an issue in many European countries; although information on the prevalence of carbapenem resistance is difficult to obtain, it appears from the outbreak literature that carbapenem resistance rates are higher in Turkey, Greece, Italy, Spain, and England, and are still rather low in Germany and in The Netherlands.

In Europe, outbreaks are especially attributable to three *A. baumannii* clones (the so-called European clones I, II, and III) [37]. Clone I was isolated in Spain, Poland, and the UK, and clone II was isolated in Italy, Spain, Portugal, France, Greece, the UK, and Turkey [10,38,39]. A third pan-European clone, clone III (LUH5875), which has probably persisted in European hospitals since the 1990s, has recently been described in France, The Netherlands, Italy, and Spain [14].

Our study revealed the distribution of four distinct *A. baumannii* epidemic clones isolated in the two periods 2004–2005 and 2008–2009. The vast majority of all isolates in both periods belonged to PFGE clone A (ST2), distributed throughout Italy and related to the European clone II. In particular, clonal subtype A1 spread in the ICU wards of Bari and Naples Federico II hospitals from 2004 to 2009. In both periods, clonal subtype A2 was found in different wards (surgery in 2004; medicine and transplant in 2008) at Gemelli hospital in Rome, and clonal subtype A3 only in the medicine ward at Bari hospital, with an increase in 2008 (nine isolates). All isolates belonging to clone B (ST1) were obtained in the hospital of Bari in both periods, whereas in Rome S. Pertini and Novara hospitals, they were isolated only in 2008 (in the second period). Clone C (ST20) was found for the first time in Italy only in 2004, but belonged to the same clonal complex (clonal complex 1) [40]. Eleven isolates of clone D (ST78) were found in three hospitals, with polyclonal spread in the Novara hospital (clones D and B) and in Naples Federico II (clones D and A). Interestingly, their detection at Naples Federico II occurred during 2008, suggesting the spread of a new *A. baumannii* epidemic clone in the same ICU, owing to colonization of patients, as already demonstrated in a previous study [41]. The polyclonal spread occurred also in Rome, especially in the second period, as clones A1, A2 and B were found in all of the Rome hospitals.

All the above data show that most of these multidrug-resistant epidemic strains were spread in the south of Italy.

In the present study, 68% of CRAB strains belonged to European clone II, suggesting its potential to be particularly successful in spreading all around Europe and Italy.

With regard to the mechanism of carbapenem resistance in the Italian isolates, neither metallo-β-lactamase activity nor the genes encoding these enzymes were detected in any of the studied isolates. In contrast, the genes encoding carbapenem-hydrolysing class D β-lactamases were found in all isolates, indicating that carbapenem resistance is caused by acquired carbapenamases.

Since 1993, the year in which the first acquired OXA enzyme was found [42], the spread of this acquired mechanism has been well documented [43], as has the role of clonal spread of the major European lineages. Our results show the presence of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$ alone and, in a few

isolates, together. The recent observation of an increase in meropenem MICs resulting from the presence of OXA-23 strains [15] was not confirmed in our isolates. The high level of carbapenem resistance in our isolates does not correlate with the presence of bla_{OXA-58}, bla_{OXA-23}, or both together: the different expression of intrinsic chromosomal $bla_{OXA-51-like}$ genes could affect the MIC values obtained (L. Pagani, personal observation).

Furthermore, the coexistence of bla_{OXA-58} and bla_{OXA-23} found in our isolates, recently reported by Higgins *et al.* [44] in a molecular epidemiology study focused on the distribution of carbapenemase genes in *A. baumannii* worldwide, has not been described in Italy before. The increased frequency of bla_{OXA-23} was attributable to the spread of this successful PFGE type A.

All CRAB isolates described in the present study showed a similar anti-biotype pattern, characterized by resistance to all of the antibiotics tested except tigecycline and colistin, which remain the drugs of choice for the treatment of infections caused by Gram-negative multidrug-resistant isolates [27,45].

In conclusion, Italian CRAB diffusion is attributable to the spread of European clone II, which has acquired OXA-23 alone or in association with OXA-58 or OXA-51. New STs have emerged in Italy, and, in our experience, PFGE and MLST, chosen for epidemiological investigation, were equally suitable: PFGE was more useful for local outbreak investigations, and MLST was convenient for establishing relatedness with clones that have spread globally [46]. Further studies to monitor resistant gene content and expression are needed.

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Transparency Declaration

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