

Molecular typing of *Acinetobacter baumannii*–*Acinetobacter calcoaceticus* complex isolates from endemic and epidemic nosocomial infections

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SUMMARY

Ribotype, biotype and resistance phenotype were used to characterize 37 *Acinetobacter baumannii*–*A. calcoaceticus* complex isolates responsible for nosocomial infections in Buenos Aires. Nineteen isolates were recovered from endemic infections at 2 hospitals and 18 represent an intensive care unit outbreak that occurred in a third hospital. By ribotyping isolates were classified into five different clones of *A. baumannii* biotype 2, 3 of *A. baumannii* biotype 9, and 3 of *Acinetobacter* genospecies 13. Combination of the three epidemiological markers permitted categorization of 18 outbreak isolates into four probable strains: 2 *A. baumannii* biotype 2, named type I, and II, and 2 *A. baumannii* biotype 9. Type I (15 isolates) was the most prevalent strain at one hospital and was responsible for the outbreak. In conclusion, combined analysis of biotypes, resistance phenotypes, and ribotypes was an accurate approach for epidemiologic investigation of *A. baumannii*. Furthermore, ribotyping discriminated *Acinetobacter* genospecies 13 isolates which were phenotypically difficult to type.

INTRODUCTION

From all DNA groups (genospecies) described within the genus *Acinetobacter*, genospecies 2 (*A. baumannii*) is the one most frequently associated with nosocomial outbreaks or cross-infection, and it poses a serious challenge to prevention of hospital infection [1–3]. *A. calcoaceticus*, *A. lwoffii* and other unnamed species such as *Acinetobacter* genospecies 3 and genospecies 13 have also been associated with nosocomial infections [2, 4–7].

Because DNA–DNA hybridization methods [8] may be impractical to categorize *Acinetobacter* DNA groups in most clinical bacteriology laboratories, a simplified phenotypical scheme was proposed for identification of clinically relevant *Acinetobacter* species [1]. In addition, biotyping through assimilation tests permits classification of *A. baumannii* into 18 biotypes [3]. Gerner-Smidt and colleagues [9] have pointed out difficulties in the discrimination of DNA groups 1, 2, 3 and 13 of the Bouvet and Jeanjean's classification [3] using the phenotypic tests

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proposed, and suggested that these four members should be referred to as the *A. calcoaceticus*–*A. baumannii* complex. These authors also suggested that ribotyping may be a good tool for taxonomic identification of *A. calcoaceticus*–*A. baumannii* complex strains [10]. Furthermore, ribotyping can provide a useful typing scheme for epidemiological studies [10, 11]. In fact, there is no generally accepted typing procedure for epidemiological studies of *Acinetobacter* isolates, although several schemes have been proposed, such as phage [2], bacteriocin [12] types, serotypes [13], plasmid [14, 15] and cell envelope protein profiles [2], and DNA restriction fragment length polymorphisms (RFLPs) determined by pulsed-field gel electrophoresis [16].

This study addresses the characterization of *Acinetobacter baumannii*–*A. calcoaceticus* complex isolates recovered from nosocomial infections which occurred at three different hospitals of Buenos Aires, Argentina. Biotype, antibiotype and ribotype were utilized as epidemiological markers to investigate the identity of *A. baumannii*–*A. calcoaceticus* complex strains.

MATERIALS AND METHODS

Bacterial strains. A total of 37 *A. calcoaceticus*–*A. baumannii* complex isolates were investigated: 19 were obtained from endemic infections occurring at the A. Lanari and the CEMIC General Hospitals, whereas another 18 were retrieved from the A. Posadas General Hospital of Buenos Aires, within a period of 2 months during an intensive care unit outbreak. All *A. calcoaceticus*–*A. baumannii* complex isolates were phenotypically characterized in our laboratory according to the Bouvet and Grimont scheme [1]. Growth tests at different temperatures were performed by inoculation of 10 ml of both Brain Heart Infusion (BHI) and Typticase Soy Broth (TSB). *A. baumannii* biotypes were established by utilization of citraconate, L-phenylalanine, levulinate, 4-hydroxybenzoate and L-tartrate as single carbon source in minimal medium [4]. The MICs against 11 antimicrobial agents were determined by the agar dilution method, using a Steers replicator device to dispense a final inoculum of 10^4 CFU/ml, according to NCCLS recommendations [17].

Ribotyping. DNA was extracted by a routine method [18]. Briefly, bacterial pellets from overnight broth cultures were suspended in Tris-HCl-EDTA buffer (50 mM-50 mM, pH 8) and treated with lysozyme (Sigma Chemical Co, St. Louis, MO) for 60 min at 37 °C (200 µg/ml, final concentration). Cells were lysed by treatment with 1% sodium dodecyl-sulphate (BRL, Life Technologies Inc., Gaithersburg, MD) and 200 µg/ml proteinase K (Sigma) at 56 °C for 60 min. RNase (Sigma) was then added to obtain a final concentration of 200 µg/ml and the tubes were further incubated for 30 min at 37 °C. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) until no proteins were detected at the interface, and then with chloroform and finally with ether. DNA was precipitated by centrifugation with sodium acetate and cold propan-2-ol, washed with 70% ethanol and suspended in 200 µl Tris-HCl-EDTA buffer (10 mM-1 mM, pH 8). DNA concentration and purity were estimated spectrophotometrically at 260/280 nm. Endonucleases *EcoRI*, *ClaI* and *SaI* (BRL) were used for DNA digestion [10], according to the procedure described by the manufacturer.

*Hind*III digestion (BRL) was applied on isolates with similar *Eco*RI ribotype. Electrophoresis was performed in a 0.8% agarose gel with 1 × TBE buffer (pH 8), overnight at 45 V. DNA was transferred to a nylon membrane (ZetaProbe GT, Bio-Rad Laboratories, Hercules, CA) according to a standard protocol using 0.5 M-NaOH–0.6 M-NaCl as transfer solution. Labelled cDNA from *Escherichia coli* 16S-23S rRNA (Boehringer Mannheim GmbH, Germany) was obtained by random priming, using digoxigenin-11-UDP and Moloney murine leukaemia virus reverse transcriptase (BRL). Prehybridization, hybridization and detection were carried out as specified in the DIG DNA Labelling and Detection Non-Radioactive Applications Manual (Boehringer). The colour reaction was stopped after 4 h. Ribotype numerical analysis was performed by UPGMA clustering (unweight pair-group method, arithmetic average) of the molecular weight of the banding patterns by means of the simple matching coefficient.

RESULTS

Biotyping and resistance phenotype. All 37 isolates (19 recovered from endemic infections and 18 from the outbreak) exhibited significant growth in BHI and TSB at 37, 41 and 44 °C within 24 h. Twenty-eight isolates produced acid from glucose, exhibited a positive reaction in Simmons citrate slants, utilized malate, malonate, β -alanine, L-ornithine, L-arginine, DL-lactate, L-tyrosine, L-leucine, ethanol, L-phenylalanine, laevulinate or 4-hydroxybenzoate, did not hydrolyse gelatin, did not utilise citraconate, L-tartrate or histamine and did not produce haemolysis on sheep blood agar. These isolates were phenotypically identified as *A. baumannii* biotype 2. The remaining nine isolates were different because they failed to assimilate both malonate and levulinate. Therefore, they were categorized as *A. baumannii* biotype 9 or *Acinetobacter* genospecies 13. Two additional isolates, that phenotypically resembled *Acinetobacter* genospecies 3 and *A. junii*, were excluded from the study because they were not considered to be the true etiological agents of the infections but only microorganisms colonizing the respiratory tract. Sixteen isolates from the outbreak were classified as *A. baumannii* biotype 2, whereas two isolates were phenotypically classified as *A. baumannii* biotype 9 (or *Acinetobacter* genospecies 13). Four resistance phenotypes were observed among the outbreak isolates (Table 1). Fifteen *A. baumannii* biotype 2 isolates showed the antibiotype A and one the antibiotype C. Those isolates classified as *A. baumannii* biotype 9 or *Acinetobacter* genospecies 13 exhibited antibiotypes B and D, respectively (Table 1).

Ribotyping. *Eco*RI ribotyping of 37 isolates identified 11 different banding patterns (Figs. 1 and 2). From 28 *A. baumannii* biotype 2 isolates *Eco*RI ribotyping demonstrated six probable sub-types, designated 1a, 1b, 1c, 1d, 1e and 1f. Ribotype 1a was observed in 15 of the 16 *A. baumannii* biotype 2 outbreak isolates. From these, one *A. baumannii* biotype 2 isolate showed an additional band of approximately 3.2 kb, which was confirmed in duplicate gels (Fig. 1A, isolate number 18). This isolate produced no additional band after DNA digestion with either *Sal*I or *Cla*I. Numerical analysis by means of the coefficient of similarity (CS) indicated that ribotypes 1a and 1b were very closely related (CS = 95.3%) and, therefore, they were considered to belong in the same clone. The

Table 1. Antimicrobial sensitivity of *A. baumannii* outbreak isolates

Isolate number	A/S	PIP	CAZ	CTX	IMP	MICs ($\mu\text{g/ml}$)								Anti-biotype
						GEN	AMI	NET	NOR	CIP	OFX			
1-3, 5-7, 9-15, 17 and 18	4	> 256	32	128	< 0.5	> 256	64	16	16	< 0.5	1	A		
4	4	> 256	4	16	< 0.5	< 0.5	< 0.5	1	2	< 0.5	< 0.5	B		
8	2	> 256	8	32	< 0.5	256	256	16	> 256	128	64	C		
16	32	> 256	16	32	< 0.5	4	4	256	> 256	256	64	D		

A/S, ampicillin/sulbactam; PIP, piperacillin; CAZ, ceftazidime; CTX, ceftaxidime; IMP, imipenem; GEN, gentamicin; AMI, amikacin; NET, netilmicin; NOR, norfloxacin; CIP, ciprofloxacin; OFX, ofloxacin.

* Phenotypically classified as either *A. baumannii* biotype 9 or *Acinetobacter* genospecies 13.

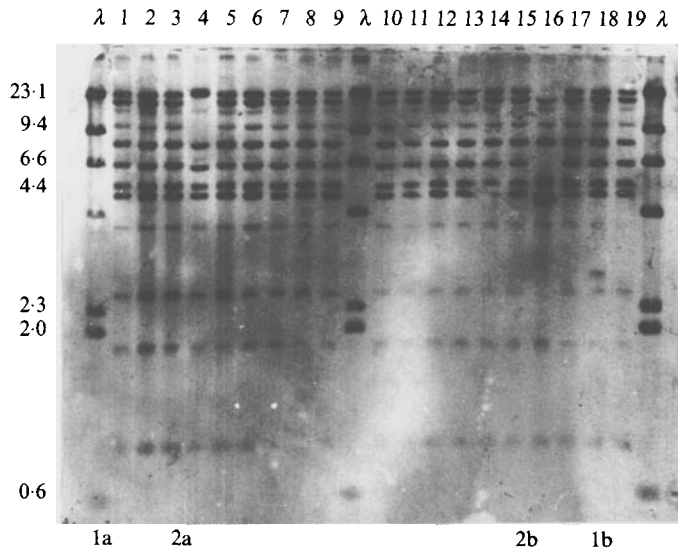


Fig. 1. *EcoRI* ribotypes of *A. baumannii*–*A. calcoaceticus* complex isolates from an outbreak. Lanes 1–18, outbreak isolates from the A. Posadas Hospital. Lane 19, isolate from another Hospital. Lanes 1–3, 5–15 and 17–19, *A. baumannii* biotype 2. Lanes 4 and 16, *A. baumannii* biotype 9. Lane λ , molecular mass marker (λ -phage DNA digested with *HindIII*).

similarity between *EcoRI* ribotypes of the endemic strains is shown in Figure 3. Ribotypes 1a, 1c and 1f of *A. baumannii* biotype 2 had a CS > 85% when compared to each other. The CS of ribotypes 1d and 1e were, however, < 85% when compared either to each other or with the above mentioned ribotypes. Ribotype 1c of isolate number 4 was subsequently confirmed in a separate gel (photograph not shown). All *A. baumannii* biotype 2 ribotypes, except ribotype 1d, were found in both Hospitals, Lanari and CEMIC. This ribotype was isolated only from a patient at the CEMIC Hospital. The *EcoRI* 1a ribotype was the one most frequently found causing endemic infections in patients at the A. Lanari Hospital. *HindIII* digestion of DNA from isolates with *EcoRI* ribotype 1a did not further discriminate any additional ribotypes.

The 9 isolates phenotypically classified as *A. baumannii* biotype 9 (or *Acinetobacter* genospecies 13) were categorized by *EcoRI* ribotyping into 6 *A. baumannii* biotype 9 and 3 *Acinetobacter* genospecies 13 isolates. *A. baumannii* biotype 9 was recognized by the characteristic presence of 0.8 kb and 5.2 kb bands and the absence of the 1.8 kb band after *EcoRI* digestion (Figs. 1 and 2), and the presence of a 3.0 kb band after *SalI* digestion. The two *EcoRI* ribotypes of *A. baumannii* biotype 9 isolates from the outbreak were designated 2a and 2b (Fig. 1). The *ClaI* fragmentation patterns of these two isolates showed dissimilar ribotypes which, in turn, were different from those from other isolates investigated. According to the banding score proposed by Gerner-Smidt [10], isolate number 4 could not be assigned to any DNA group after *ClaI* ribotyping although it was easily classified as *Acinetobacter* genospecies 2 by *EcoRI* ribotyping. Discordance in the results obtained after digestion with *ClaI* or *EcoRI* were also observed in two other isolates from endemic infections. The four *A. baumannii* biotype 9

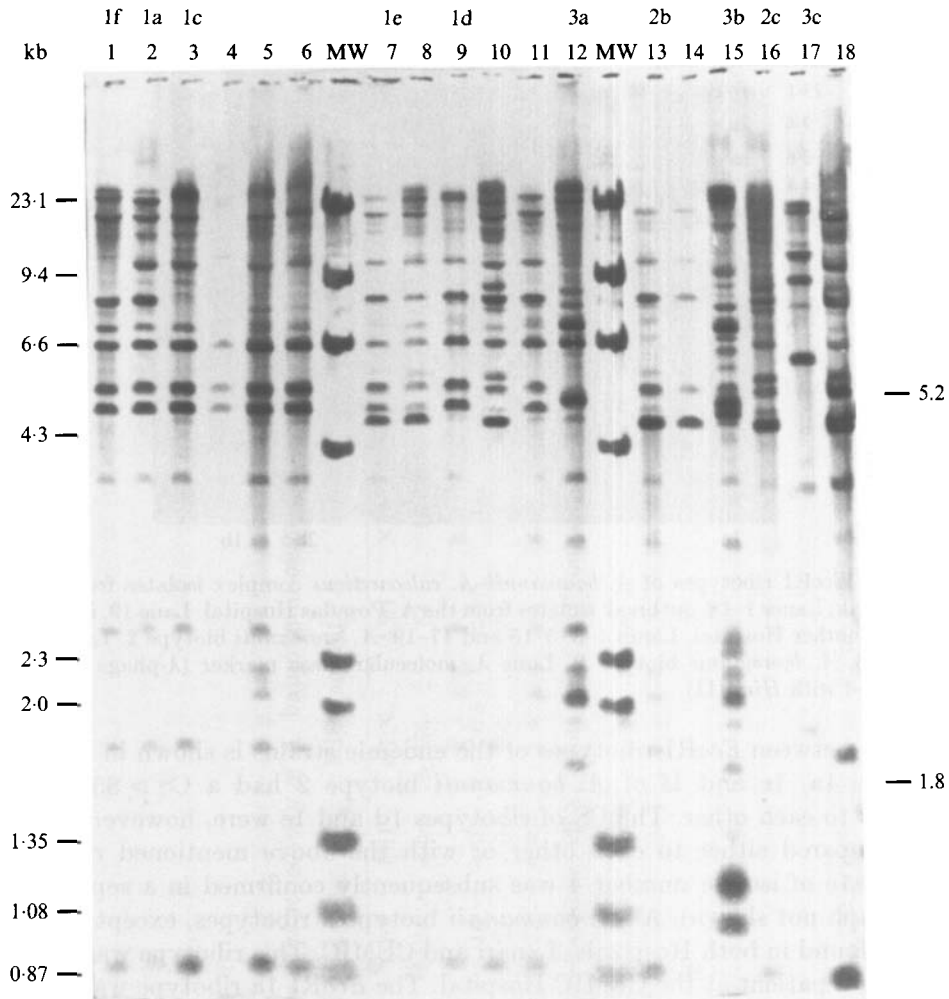


Fig. 2. *EcoRI* ribotypes of *A. baumannii*-*A. calcoaceticus* complex isolates recovered from endemic infections. Lanes 1-11, *A. baumannii* biotype 2. Lanes 13, 14, 16 and 18, *A. baumannii* biotype 9. Lanes 12, 15 and 17, *Acinetobacter* genospecies 13. Lanes 1-3, 7, 11 and 14, clinical isolates from the Lanari Hospital. Lanes 4-6, 8-10, 12-14 and 16-18, clinical isolates from the CEMIC Hospital. Lane MW, λ -phage DNA digested with *HindIII* and phage $\phi \times 174$ DNA digested with *HaeIII*.

isolates recovered from endemic infections exhibited ribotypes 2b and 2c (Fig. 2), which clustered together with a CS = 83.0% between these ribotypes, and of 70.0% when compared with *A. baumannii* biotype 2 ribotypes. The rDNA RFLP after *EcoRI* digestion of the three *Acinetobacter* genospecies 13 isolates showed the lack of the 0.8 kb band. Two of them exhibited the 1.8, 2.0 and 7.8 kb scored bands proposed by Gerner-Smidt [10] for this *Acinetobacter* DNA group (Fig. 2 lanes 12 and 15). The third isolate was classified as *Acinetobacter* genospecies 13 due to the presence of the 3.9 and 5.4 Kb scored bands [10] (Fig. 2, lane 17). When all band patterns were considered, these three isolates were categorized in ribotypes 3a, 3b and 3c.

Combination of epidemiological markers. Combination of three epidemiological

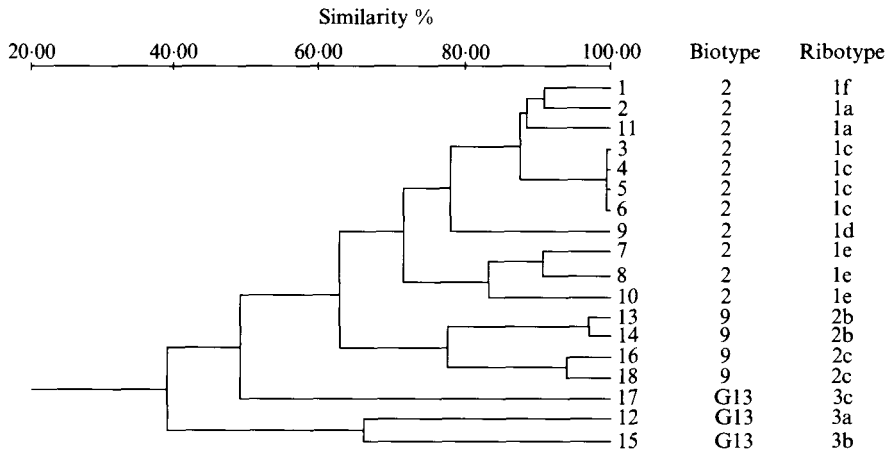


Fig. 3. UPGMA clustering dendrogram of *Eco*RI ribotypes of *A. baumannii*–*A. calcoaceticus* complex isolates from endemic infections. Isolates numbered as shown in Fig. 2.

Table 2. *A. baumannii* strains isolated from an intensive care unit outbreak

Typing system	Strains			
	Type I	Type II	Type III	Type IV
Biotype	<i>A. baumannii</i> biotype 2	<i>A. baumannii</i> biotype 2	<i>A. baumannii</i> biotype 9	<i>A. baumannii</i> biotype 9
Ribotype	1a	1a	2a	2b
Antibiotype	A	C	B	D
Isolate number*	1–3, 5–7, 9, 10–15, 17 and 18	8	4	16

* Same numbers as those in Fig. 1.

markers was utilized to characterize single strains. Biotype, resistance phenotype and ribotype categorized the 18 *A. baumannii* isolates from the outbreak into four probable strains (Table 2). *A. baumannii* biotype 2 strains were defined as follows: strain type I (ribotype 1a, antibiotype A) (15 isolates) and strain type II (ribotype 1a, antibiotype C) (1 isolate). According to their ribotypes these two strains represented one single clone, although strain II exhibited resistance to fluoroquinolones, which was not observed in any of the strain I isolates. In three patients with two simultaneous isolates from different infection sites, strain type I was recovered from both sites: (i) patient 1, from cerebrospinal fluid and bronchoalveolar secretion cultures; (ii) patient 2, from blood and bronchoalveolar secretion cultures, and (iii) patient 3, from the proximal skin insertion segment and the distal end of a catheter as well as from blood cultures.

DISCUSSION

The *A. calcoaceticus*–*A. baumannii* complex is frequently involved in nosocomial infections in intensive care units of Argentina. In this study *A. baumannii* biotype 2, *A. baumannii* biotype 9 and *Acinetobacter* genospecies 13 were recovered from endemic infections. The occurrence of *A. baumannii* biotypes isolated from clinical

specimens agreed with other studies from diverse geographic areas. In France, Joly-Guillou and colleagues found that biotypes 1, 2, 6 and 9 are frequently isolated from patients and the hospital environment [7]. Other reports indicated that biotypes 2, 6 and 9 are commonly associated with nosocomial outbreaks [6, 10].

The use of the phenotypic scheme proposed by Bouvet and Grimont [1, 3] to identify clinical *Acinetobacter* isolates met with problems similar to those reported by Gerner-Smidt and colleagues [9], particularly in the discrimination of *A. baumannii* biotype 9, which closely resembles *Acinetobacter* genospecies 13. Gerner-Smidt [10] proposed ribotyping after *EcoRI* genomic DNA digestion as a good method to discriminate *A. baumannii*–*A. calcoaceticus* complex species and recommended ribotyping after digestion with *SalI* or *ClaI* to confirm the *EcoRI* ribotype. We found that ribotyping was an accurate procedure to identify both genospecies, *A. baumannii* and *Acinetobacter* genospecies 13, primarily by the presence or absence of the 0.8 kb band, among other bands, after *EcoRI* digestion. In general, *Acinetobacter* DNA groups categorized by *EcoRI* ribotyping were consistent with those observed by *SalI* ribotyping. Conversely, the patterns obtained with *ClaI* rDNA RFLP were difficult to interpret by the Gerner-Smidt scoring system [10]. Ribotyping permitted recognition of different clones within these species. By means of this typing system it was possible to categorize the 28 *A. baumannii* biotype 2 isolates into 5 different *EcoRI* ribotypes, and the numerical analysis of banding patterns by the UPGMA clustering method showed that they were more related to each other than to the *A. baumannii* biotype 9 ribotypes. Furthermore, ribotyping showed that *EcoRI* ribotype 1a, the one most frequently found causing endemic infections in patients of the A. Lanari Hospital and responsible for Hospital Posadas intensive care unit outbreak, belonged in the same clone as *A. baumannii* ATCC 19606 studied by Gerner-Smidt [10]. In addition, ribotyping permitted categorization of the three *Acinetobacter* genospecies 13 isolates into a DNA group different from that of *A. baumannii*, as shown by UPGMA clustering.

With regard to the relative value of the methods utilized as taxonomic identification tools, phenotyping was adequate to classify *A. baumannii* biotype 2 isolates. This identification was later confirmed by ribotyping. The fact that both phenotyping and ribotyping were required to differentiate *A. baumannii* biotype 9 from *Acinetobacter* genospecies 13 isolates is an important issue to be considered when addressing the role of the clinical microbiology laboratory in infection control programmes. Clinical laboratory findings usually provide the first evidence of potential dissemination of bacteria from patient to patient, from patients to nosocomial personnel and/or the environment, or vice versa. Frequently, for most species, bacterial phenotypic features and antimicrobial susceptibility profiles lead to the suspicion of nosocomial spread of a common microorganism. In those circumstances, molecular techniques sustain the epidemiological investigation rather than inspiring it [19]. In the particular case of *A. calcoaceticus*–*A. baumannii* complex infections, ribotyping may overcome the weaknesses of phenotypic assays for appropriate identification of *Acinetobacter* isolates up to the DNA group level.

On the basis of data presented here and elsewhere phenotypic methods such as biotyping and antibiogram typing do not appear powerful enough to be used alone

in epidemiological studies of *Acinetobacter* infections [6, 16]. Other authors maintain that phage typing may not be a good choice either because approximately 30% of the strains involved in nosocomial infections are non-typable by this procedure [2, 7] and furthermore, many laboratories do not possess the necessary phage sets. Determination of the cell envelope protein profile is available in many laboratories, but it may lack the discrimination power required for the task. In this regard, homogeneous profiles among species of *Acinetobacter* isolated from the same hospital have been reported [4]. Among molecular typing methods, RFLP profile analysis using low-frequency-cleaving endonucleases is well-suited for discriminating epidemiologically unrelated strains. Although it may be common in developed countries, not many laboratories worldwide have pulsed-field gel electrophoresis equipment and computerized scanners available [16].

Ribotyping seems to be a good approach not only to type *A. calcoaceticus*–*A. baumannii* complex isolates but also to discriminate isolates into clones [11]. Moreover, ribotyping can be considered particularly more dependable than other methods in the assessment of clonality among nosocomial isolates collected over long time periods, because ribosomal DNA patterns are insensitive to antibiotic pressure. All the isolates investigated here were submitted to our laboratory as *A. baumannii* and ribotyping allowed detection of eight different clones of *A. baumannii* and three different clones of *Acinetobacter* genospecies 13. In favour of antimicrobial susceptibility testing, it should be noted that among the *A. baumannii* biotype 2-ribotype 1a isolates investigated, the antibiotype permitted delineation a probable different strain originated from the same clone as the strain named type II (ribotype 1a, antibiotype C). This finding suggests that the combination of ribotyping with other molecular epidemiological markers may be the most suitable choice to subclassify ribotype 1a isolates. In this particular case the plasmid profile was of no use because all outbreak ribotype 1a isolates exhibited the same plasmid profile (unpublished data). In any event, a combination of two or more epidemiological markers is required to identify single strains [6, 20]. The usefulness of modern molecular markers such as ribotyping, PFGE and fingerprinting by arbitrary primers as epidemiological tools remain to be established [21].

In conclusion, none of the markers utilized in this study appears suitable alone for epidemiological investigation of *A. baumannii*–*A. calcoaceticus* complex infections. Combination of epidemiological markers, including biotype, resistance phenotype, and ribotype, however, allowed characterization of the strain responsible for an intensive care outbreak. Moreover, the use of molecular markers also demonstrated that the frequently detected resistance to norfloxacin, in association or not with resistance to other fluoroquinolones, emerged in multiple *A. baumannii* biotypes and clones.

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