Association of Plasmid Typing to Biotyping and Antibiotyping in the Characterization of Outbreaks by *Acinetobacter baumannii.*

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ABSTRACT

During an outbreak at an University Hospital, from April to September, in 1994, sixteen strains of Acinetobacter baumannii *were isolated from patients and one strain from an enteral solution. We afterwards analyzed the outbreak by means of plasmid typing, antibiotic resistance typing and biotyping. Two main plasmid profiles were identified. Twelve strains belonged to biotype 2, and five to biotype 19. Susceptibility to amikacin and to carbenicillin allowed classification of the strains into two groups. The results show that association of those three typing methods allowed the differentiation of what was at first considered as a single outbreak into two apparently unrelated outbreaks.*

Key words: *Acinetobacter baumannii*; outbreak; plasmid, biotyping.

INTRODUCTION

Strains of the genus *Acinetobacter* have recently been recognized as agents of nosocomial infections that represent a significant problem in many countries (Muller-Serieys *et al.*, 1989; Ramphal & Kluge, 1979). This genus comprises at least 17 genomic species (Tjernberg & Ursing, 1989), and the species *A. baumannii* has been the one most frequently isolated from patients bearing hospital-acquired *Acinetobacter* infections (Beck-Sagué *et al.*, 1990; Oliveira *et al.*, 1993). The route of transmission in most outbreaks by these bacteria includes the contamination of devices such as intravenous catheters, prosthetic valves, respiratory equipment used in conjunction with endotracheal tubes or tracheotomy, and also bedding material (Beck-Sagué *et al.*, 1990 ; Harststein *et al.*, 1988 ; Sherertz & Sullivan, 1985; Weernink *et al.*, 1995).

Various typing methods have been used for the identification of pathogenic isolates of *Acinetobacter*, such as biotyping (Oliveira *et al.*, 1993; Bouvet & Grimont, 1987; Bouvet *et al.*, 1990), serotyping (Traub & Spohr, 1994), antibiotic resistance typing (Vila *et al.*, 1989), phage typing (Bouvet *et al.*, 1990; Santos-Ferreira *et al.*, 1984), and envelope protein profiles (Bouvet *et al.*, 1990; Dijkshoorn *et al.*, 1989). However, all these methods evaluate the expression of phenotypic characters which can be affected by many environmental factors; genotyping methods, on the other hand, are more stable but more expensive and time-consuming. Among the genotyping methods, plasmid typing (Vila *et al.*, 1989; Seifert *et al.*, 1994a), ribotyping (Gerner-Smidt, 1992; Seifert & Gerner-Smidt, 1995), analysis of restriction fragment length polymorphisms by pulsed-field gel electrophoresis (Seifert & Gerner-Smidt, 1995; Gouby *et al.*, 1992), and PCR fingerprinting (Graser *et al.*, 1993; Reboli *et al.*, 1994) have been used for the identification of *Acinetobacter*. Since biotyping and antibiotic resistance typing are routinely performed at

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hospital laboratories, and given that plasmid typing is the easiest and least expensive of the genotyping methods, the purpose of this study was to determine whether an association of these three methods could allow an adequate epidemiologic characterization of an outbreak of infection by *Acinetobacter sp*.

MATERIALS AND METHODS

Bacterial strains: Seventeen *Acinetobacter* strains were isolated during an outbreak which occurred from April to September, in 1994, at the University Hospital, in Londrina, Brazil. Sexteen of them were isolated from clinical specimens of 14 hospitalized patients in intensive care unit, one isolated from nursery patient, and another strain from an enteral solution. The isolates were identified as *A. baumannii* according to Bouvet & Grimond (1987) and maintained either on Luria Bertani (LB) broth at -20°C or on LB slants agar at room temperature. The strain ATCC 14606 was used as control.

Biotying of *A. baumannii* strains: Utilization of six carbon sources (citraconate, levulinate, L-phenylalanine, phenylacetate, 4-hydroxybenzoate and L-tartarate), allowed the identification of biotypes, as described by Bouvet & Grimond (1987).

Antibiotic susceptibility: Susceptibility to 15 antibiotics was determined by the disk diffusion method (Bauer *et al.*, 1966), on Müeller-Hinton agar (Difco). The following disks were used: Nalidixic acid (30 μg); Amikacin (30 μg), Ampicillin (10 μg); Carbenicillin (100 μg); Cephalothin (30 μg); Cefotaxime (30 μg); Ceftazidime (30 μg); Chloranphenicol (30 μg); Gentamicin (10 μg); Imipenem (10 μg); Perfloxacin (5 μg); Tobramycin (10 μg); Sulfazotrin (25 μg); Tetracycline (30 μg). Additionaly, the MIC was determined by dilution test agar method according to Sahm & Washington II (1991).

Plasmid analysis: Plasmid DNA was isolated from the 17 strains by the alkaline method of Kado & Liu (1981), with some modifications. Cells were grown overnight in 3 ml of LB broth medium at 37°C and 1.5 ml was spun down at 10.000 x g for 1 min. The pellet obtained was washed in 1 ml of TE buffer (50 mM Tris-HCl, 10 mM EDTA - pH 8.0). After centrifugation the cells were resuspended in 50 μl of TE, and 400 μl of lysing solution (50 mM Tris and 3% SDS - pH 12.45) were added. The solution was mixed by brief agitation and incubated in a water bath at 60°C for 30 min. The lysate was subsequently incubated on ice water, and 20 μl of 2M Tris-HCl (pH 7.0) were added. The extraction was made by addition of an equal volume of phenol-chloroform solution. After centrifugation as above for 10 min, 60 μl of aqueous phase were mixed with loading buffer and applied to a vertical agarose gel (0.7%) for electrophoresis at 60 V. The plasmids were also extracted as described by Harstein *et al.* (1990). The isolates were grown on LB agar plates at 37°C for 24 hours. Cells from one quarter of the plate were resuspended twice in 1.0 ml of 2.5 M NaCl, 10 mM EDTA (pH 8.0). After centrifugation, 900 μl of 20% sucrose, 50 mM Tris, 10 mM EDTA (pH8.0) and 200 μl of lysozyme (10 mg/ml) were added to the pellet and incubated at 30°C for 30 min. After incubation, 500 μl of 2.5 M NaCl, 10 mM EDTA (pH 8.0) and a lysis solution containing 250 μl of 0.5% mixed alkyltrimethilamonium bromide (ATAB) and 50 μl of 10% Triton X100 were added to each test tube. The resulting lysate was incubated in a water bath at 56ºC for 15 min and then centrifuged at 10.000 x g for 30 min. To the supernatant was added 1 μl of RNAse solution (10 mg/ml) and incubated at 37ºC for 30 min. Protein was eliminated by two washes with phenol-chloroform-isoamyl alchool (25:24:1) solution. Finally, plasmid DNA was precipitated by addition of an equal volume of ice-cold isopropanol. The precipitate was spun down for 20 min at 10.000 x g, dried and then resuspended in 60 μl of the TE buffer (10 mM Tris, 1mM EDTA - pH 8.0). Electrophoresis was performed on a horizontal gel containing 0.8% agarose in TEB. Samples (30 μl) were mixed with running dye buffer (8 μl), loaded onto the agarose gel (1%), and run for 12h at 40 V. Gels were stained with ethidium bromide and photographed under a U.V. lamp. The following

plasmids were used as markers of molecular mass: pSa (23 MDa), RP4 (34 MDa), JPN11 (66 Mda), R27 (110 Mda), plus the standard plasmids from *E.coli* V517.

RESULTS

Antibiotic susceptibility: All studied *A.baumannii* strains were susceptible to imipenem (≤4μg/ml), and resistant to ampicillin, cephalothin, chloramphenicol, ceftazidime (≥32μg/ml), and trimethoprin, gentamicin (≥16μg/ml). The *A. baumannii* strains that showed differents antibiotic susceptibilities pattern, are showed at table I. Excepting imipenem, the matrix of 15 antimicrobial agents versus 17 strains, with 255 entries, presented 195 (76.5%) occurrences of resistance, 38 (14.9%) occurrences of intermediate resistance, and 22 (8.6%) occurrences of susceptibility; therefore, most of the strains were resistant to most of the antibiotics tested. Fifty percent (11/22) of the occurrences of susceptibility corresponded to tetracycline, that is, 64.7% (11/17) of the strains were susceptible to tetracycline. Resistance to amikacin and to carbenicillin allowed the classification of strains into two patterns. Strains that were either susceptible or intermediate-resistant to amikacin and carbenicillin were classified as belonging to pattern A; strains that were resistant to those antibiotics were classified as pattern B (Table 1).

Biotyping: Two biotypes of *A. baumannii*, 2 and 19, were isolated from patients; the strain isolated from an enteral solution belonged to biotype 2 (Table 2).

Table 1. Antibiotic Susceptibilities of *Acinetobacter baumannii* strains.

Strain	MIC concentration (μg/ml) / Antibiotic Susceptibilities pattern								
	AM	**CAB**	**CFT**	**CIP**	**NAL**	**PEF**	**SUL**	**TOB**	**TET**
01	>64 (R)	>64 (R)	>64 (R)	1<4 (I)	<16 (S)	4<16 (I)	>512 (R)	>16 (R)	<4 (S)
06	<16 (S)	16<64 (I)	>64 (R)	1<4 (I)	<16 (S)	4<16 (I)	>512 (R)	>16 (R)	<4 (S)
09	<16 (S)	16<64 (I)	>64 (R)	1<4 (I)	<16 (S)	4<16 (I)	>512 (R)	>16 (R)	<4 (S)
10	16<64 (I)	16<64 (I)	>64 (R)	1<4 (I)	>32 (R)	4<16 (I)	>512 (R)	>16 (R)	<4 (S)
11	16<64 (I)	16<64 (I)	>64 (R)	1<4 (I)	>32 (R)	4<16 (I)	>512 (R)	>16 (R)	<4 (S)
12	<16 (S)	16<64 (I)	>64 (R)	1<4 (I)	<16 (S)	4<16 (I)	256<512 (I)	>16 (R)	<4 (S)
13	<16 (S)	16<64 (I)	8<64 (I)	<1 (S)	<16 (S)	<4 (S)	256<512 (I)	4<16 (I)	<4 (S)
15	>64 (R)	>64 (R)	>64 (R)	1<4 (I)	>32 (R)	4<16 (I)	256<512 (I)	4<16 (I)	<4 (S)
16	>64 (R)	>64 (R)	>64 (R)	1<4 (I)	16<32 (I)	4<16 (I)	>512 (R)	>16 (R)	<4 (S)
17	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>32 (R)	>16 (R)	>512 (R)	>16 (R)	<4 (S)
18	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>32 (R)	>16 (R)	>512 (R)	>16 (R)	<4 (S)
19	>64 (R)	>64 (R)	>64 (R)	1<4 (I)	>32 (R)	4<16 (I)	>512 (R)	4<16 (I)	>16 (R)
21	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>32 (R)	>16 (R)	>512 (R)	>16 (R)	4<16 (I)
22	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>32 (R)	>16 (R)	>512 (R)	>16 (R)	>16 (R)
23	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>32 (R)	>16 (R)	>512 (R)	>16 (R)	>16 (R)
24	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>32 (R)	>16 (R)	>512 (R)	>16 (R)	>16 (R)
En	>64 (R)	>64 (R)	>64 (R)	1<4 (I)	16<32 (I)	4<16 (I)	>512 (R)	>16 (R)	>16 (R)

AM=amikacin; **CAB=**carbenicillin; **CFT=**cefotaxime; **CIP=**Ciprofloxacin; **NAL=**nalidixic acid; **PEF=**perfloxacim; **SFZ=**sulfametoxazole; **TET=**tetracyclin; **TB=**tobramycin.
R=Resistent; **I=**Intermediate; **S=**Susceptible.
En=strain isolated from enteral solution

Plasmid analysis: Plasmids were found in 15 (88.2 %) strains with an average of 3 plasmids per strains; their relative molecular mass ranged from 2 to 66 MDa (Table 1). No plasmids were

found in 2 (11.8%) strains. Figs.1A and 2 show the profiles of plasmids isolated from strains of *A. baumannii* by the method of Kado & Liu (1981). Fig. 1B shows low molecular mass plasmids isolated from strains of *A. baumannii* by the method of Harstein *et al.* (1990). Based on the detection of the 66 MDa plasmid, two plasmid profiles were assigned to the *A. baumannii* strains: plasmid profile I was assigned to strains carrying the 66 MDa plasmid, and profile II was assigned to strains carrying only low (less than 13 Mda) molecular mass plasmids.

Typing of *A. baumannii:* Table 2 presents a correlation among the typing results, sampling site, and period of strain isolation. Six strains were isolated during April, five of them from catheter tips and one from skin secretion; all those strains belonged to biotype 2 and, with the single exception of one plasmidless strain, they all presented plasmid profile II (absence of the 66 Mda plasmid) and antibiotic resistance pattern A. Eleven strains isolated from June to September belonged to biotypes 2 and 19 and, excepting one plasmidless strain, all of them presented plasmid profile I(presence of the 66 Mda plasmid) and antibiotic resistance pattern B. The ratio of strains isolated from skin to isolates from catheter tips changed from 0.2 (1/5) during April to 1.2 (6/5) during June to September.

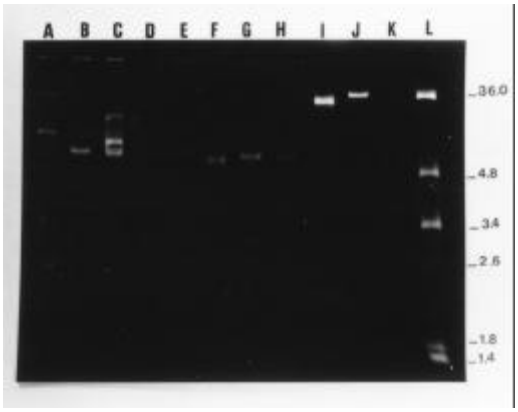

Fig. 1A. Agarose gel electrophoresis of *Acinetobacter baumannii* plasmid DNA extracted by the method of Kado & Liu (1981). Lane A: strain from enteral solution; Lanes B to H: strains 1,6,9,10,11,12, and 13 respectively; Lanes I to L, standard plasmids: RP4 (34MDa), JPN11 (66 MDa), R27 (110MDa), V517 (standard plasmids from *Escherichia coli* 517).

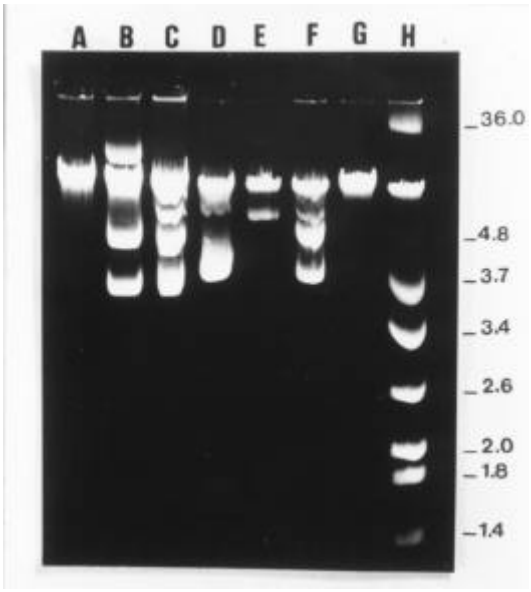

Fig. 1B. Agarose gel electrophoresis of *A. baumannii* plasmid DNA extracted by the method of Harstein *et al.* (1990). Lane B: strain from enteral solution; Lanes A, and C to G: strains 1,6,9,10,11,12, and 13 respectively. Lane H: molecular mass marker from *E. coli* V517

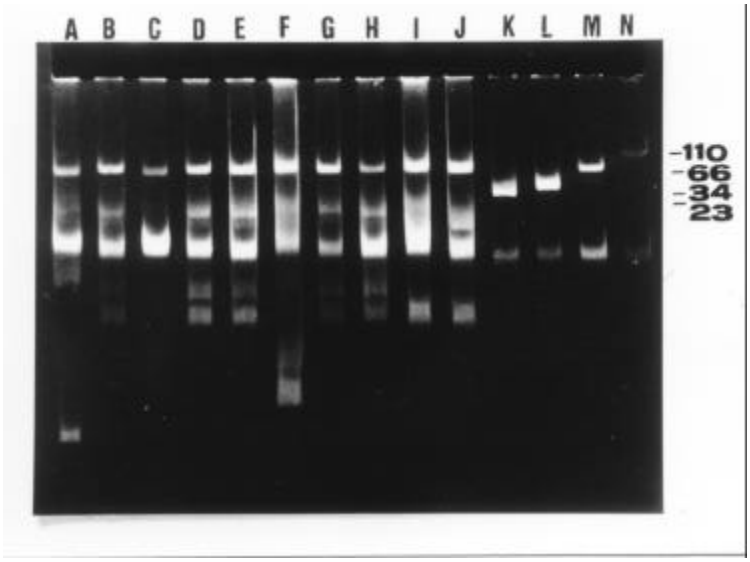

FIG. 2. Agarose gel electrophoresis of *Acinetobacter baumannii* plasmid DNA extracted by the method of Kado & Liu (1981). Lane A: strain from enteral solution; Lanes B to J: strains 15 to 19, and 21 to 24, respectively; Lanes K to N, standard plasmids: pSa (23 MDa), RP4 (34MDa), JPN11 (66 MDa), R27 (110MDa).

Table 2. Typing of 17 *Acinetobacter baumannii* strains isolated from 15 patients bearing hospital acquired infection and from one enteral solution.

Strain number	Isolated in mo/yr	Sample Site	Biotype [a]	Antibiotic susceptibility pattern [b]	Plasmid Profile/ Molecular mass
01	4/94	catheter tip	2	B	–
06	4/94	catheter tip	2	A	II / 13, 04, 03
09	4/94	catheter tip	2	A	II / 05, 04, 03
10	4/94	catheter tip	2	A	II / 05, 03
11	4/94	catheter tip	2	A	II / 13, 05
12	4/94	skin	2	A	II / 05, 04, 03
13	6/94	eyes	2	A	–
15	6/94	catheter tip	19	B	I / 66, 13
16	6/94	catheter tip	19	B	I / 66
17	6/94	skin	19	B	I / 66, 13, 05, 04
18	7/94	catheter tip	2	B	I / 66, 13, 05, 04
19	6/94	skin	19	B	I / 66, 13, 03, 02
21[c]	8/94	skin	2	B	I / 66, 13, 05
22[c]	9/94	skin	2	B	I / 66, 13, 05, 04
23	9/94	skin	19	B	I / 66, 13, 04
24	9/94	catheter tip	2	B	I / 66, 13, 04
En	6/94	enteral solution	2	B	I / 66, 13, 02
ATCC	–	padrão	19	–	–

En - strain isolated from enteral solution

[a] Biotype as determined by Bouvet & Grimont, 1987.

[b] **Pattern A:** Susceptible or intermediate-resistant to Carbenicillin and Amikacin;
Pattern B: Resistant to Carbenicillin and Amikacin; ATCC 14606: control strain

[c] Same patient

DISCUSSION

Strains of *A. baumannii* are a frequent source of hospital-acquired infections (Beck-Sagué *et al.*, 1990; Harststein *et al.*, 1988). In Brazil, *A.baumannii* was the most prevalent species (81.6%) isolated during 1990-1991 at hospitals in Ribeirão Preto City, São Paulo State (Oliveira *et al.*, 1993). All the strains of *Acinetobacter* analyzed in this study, which were isolated during an outbreak that occurred in 1994 at the University Hospital in Londrina, Paraná State, were identified as *A.baumannii.* Since *Acinetobacter* sp has become more prevalent and important as a cause of hospital-acquired infections, a simple method for the epidemiologic characterization of *Acinetobacter* sp outbreaks should be important for the monitoring and control of such outbreaks. The association of three typing methods employed in this study allowed an adequate characterization of an outbreak by *A.baumannii*. Antibiotyping and biotyping are largely used by clinical microbiology laboratories for the epidemiologic typing of *A.baumannii*, but their discriminatory power is low because the majority of clinical isolates are resistant to multiple antibiotics and belong to few biotypes (Dijkshoorn *et al.*, 1993; Seifert *et al.*, 1994b). In agreement with those previous observations, only two biotypes and two antibiotypes were distinguished in this study, and these two markers did not always correlate. However, the association of plasmid profiles with biotypes and antibiotypes allowed the identification of two apparently unrelated outbreaks by *A.baumannii*, in agreement with the suggestion by Seifert et al. that plasmid profile analysis could be useful in epidemiological typing of *A.baumannii* (Seifert *et al.*, 1994a; Seifert *et al.*, 1994b). The outbreak that occurred in April was caused by strains that did not carry the 66 Mda plasmid (profile II) and belonged to biotype 2 and antibiotype A; the single exception was a plasmidless strain that presented antibiotype pattern B. On the other hand, the outbreak that occurred from June to September was caused by strains that carried the 66 MDa plasmid (profile I) and presented antibiotype pattern B. These results suggest that the two outbreaks were caused by two different clusters of *A.baumannii*. However, the possibility cannot be excluded that some strains which caused the first outbreak subsequently acquired the 66 MDa plasmid and participated in the second outbreak (Fig 2). Because the second outbreak occurred during the winter months, and the ratio of skin isolates to catheter tips isolates increased from 0.2 (1/5) in April to 1.2 (6/5) in June to September (Table II), it is possible that another source of infection participated in the second outbreak; this observation might be related to previous reports that contaminated bedding material can be important sources of *Acinetobacter* sp (Sherertz & Sullivan, 1985; Seifert *et al.*, 1994b; Bergogne-Bérézin & Towner, 1996) and corroborates the hypothesis that the second outbreak was caused by a cluster of *A. baumannii* distinct from the first one.

Since all strains, including the two strains that supposedly carry no plasmids, presented multiresistance to drugs, some of the resistance markers of the *A.baumannii* possibly are chromosomally located, in agreement with previous studies (Bergogne-Bérézin & Towner, 1996). Nevertheless, 81.8% (18/22) of the few (22/255) occurrences of susceptibility corresponded to strains not harboring the 66 Mda plasmid, which suggests that it participates in the determination of antibiotic resistance. The method of Harstein et al. allowed a better discrimination of the low molecular mass plasmids than the method of Kado & Liu (Fig. 1A and 1B), but no unique correlation between these plasmids and resistance could be ascertained.

The results of this study show that the association of biotyping, antibiotic resistance typing, and plasmid profiles allowed the differentiation of two outbreaks by *A. baumannii* that occurred one shortly after the other and that were at first considered to be a single outbreak, suggesting that these association of methods could routinely be applied for the initial epidemiologic characterization of outbreaks by these bacteria and for monitoring their dissemination in the hospital environment.

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RESUMO

Durante um surto ocorrido de abril a setembro de 1994 em um Hospital Universitário, dezesseis cepas de *Acinetobacter baumannii* foram isoladas de pacientes e uma de solução enteral. Nós posteriormente analizamos as cepas isoladas durante o surto pelos seguintes métodos de tipagem : perfil de DNA plasmidial, perfil de antibiograma e biotipagem. Dois padrões de tipagem foram identificados pela análise do perfil plasmidial. Doze cepas foram caracterizadas como sendo do biotipo 2, e cinco do biotipo 19. O padrão de sensibilidade a amicacina e a carbenicilina possibilitou a classificação das cepas em dois grupos. Os resultados demonstraram que estes três métodos de tipagem associados possibilitaram a diferenciação do que primeiramente foi considerado como um único surto, em dois surtos aparentemente não relacionados.

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