IDENTIFICATION AND TYPING OF BACTERIA

Correlation of six methods for typing nosocomial isolates of *Acinetobacter baumannii*

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Summary. A comparative study of biotyping, antimicrobial susceptibility, whole-cell protein analysis, plasmid analysis, pulsed-field gel electrophoresis of chromosomal DNA and polymerase chain reaction with arbitrary primers of *Acinetobacter baumannii* isolates from three large hospitals was performed to determine the best markers for epidemiological purposes. Ninety-two isolates were included: 38 belonged to a previously described outbreak and 54 were randomly selected from sporadic cases of infection. Biotyping, whole-cell protein and plasmid analysis were the least discriminatory methods, whereas antimicrobial susceptibility and polymerase chain reaction with arbitrary primers showed moderate discriminatory power. Typing based on pulsed-field gel electrophoresis of chromosomal DNA appeared to be the best discriminatory method (discrimination index of 0.9623). The addition of polymerase chain reaction with arbitrary primers or antimicrobial susceptibility to pulsed-field gel electrophoresis of chromosomal DNA did not further increase the discriminatory power.

Introduction

In recent years, outbreaks of infection caused by *Acinetobacter* spp. have become a growing concern in hospitals.^{1–3} *Acinetobacter* spp. are widely distributed in the hospital environment and have been isolated from various inanimate sources such as ventilatory equipment, blood collection tubes, mattresses and plastic urinals. However, it seems that the skin of patients and staff is the most likely source for the majority of outbreaks of nosocomial acinetobacter infection.^{4–5}

Acinetobacter baumannii is the species most often associated with nosocomial infection.⁶ This organism is capable of causing life-threatening infections including bacteraemia, pneumonia and meningitis.^{7–9} The increasing importance of infections caused by *Acinetobacter* spp., and the multi-resistance of the strains involved,^{10–11} render studies of epidemiology and antibiotic resistance necessary for the prevention of further infections with this organism. There is no generally accepted typing scheme for epidemiological studies of *Acinetobacter* isolates, although various methods have been used. They include phage typing,¹² biotyping,⁶ determination of antibiotic susceptibility

patterns,^{11–13} whole-cell^{14–15} and cell envelope¹⁶ protein electrophoresis, plasmid analysis,^{3,17,18} ribotyping,^{19,20} polymerase chain reaction fingerprinting²¹ and DNA restriction fragment length polymorphism determined by pulsed-field gel electrophoresis (PFGE).^{22,23}

The purpose of this study was to describe and compare the results obtained with several of these methods—biotyping, antimicrobial susceptibility, whole-cell protein, plasmid analysis, PFGE of chromosomal DNA and polymerase chain reaction with arbitrary primers (AP-PCR)—to analyse 92 isolates of *A. baumannii* from three hospitals in Barcelona, obtained during a 3-year period.

Materials and methods

Bacterial strains

A total of 92 isolates of *A. baumannii* from different clinical specimens obtained from patients admitted to three Barcelona hospitals was examined; 48 isolates came from Hospital Clínico, 19 from Hospital de la Santa Cruz y San Pablo and 25 from Hospital Valle de Hebrón. Thirty-eight of the isolates belonged to a previously defined outbreak in the Hospital Clínico during the period Sept. 1988–Aug. 1989;³ the re-

maining 54 isolates were randomly selected from sporadic cases of *A. baumannii* infections from the three above-mentioned hospitals.

Biotyping

Isolates were identified as *A. baumannii* by standard biochemical procedures and the API 20NE system (bioMérieux). The biotype was determined by means of the utilisation of six carbon sources (levulinate, citraconate, L-phenylalanine, L-phenylacetate, 4-hydroxybenzoate, L-tartrate), as described previously.⁶

Strains of each biotype (kindly provided by Dr P. Bouvet Unité des Entérobactéries, Institut Pasteur, Paris, France) were used as controls.

Antimicrobial susceptibility

Susceptibility to antimicrobial agents was determined by the Bauer disk diffusion method.²⁴ Disks were obtained from BBL (Becton Dickinson Microbiology Systems Cockeysville, MD, USA); ampicillin 10 µg; mezlocillin 75 µg; piperacillin 100 µg; cefotaxime 30 µg; cefoxitin 30 µg; ceftriaxone 30 µg; cefuroxime 30 µg; cefalothin 30 µg; chloramphenicol 30 µg; gentamicin 10 µg; netilmicin 30 µg; tobramycin 10 µg; amikacin 30 µg; ciprofloxacin 5 µg and imipenem 10 µg.

Whole-cell protein electrophoresis

Strains were grown overnight at 37°C in brain-heart infusion broth. Cells were collected by centrifugation and washed twice in phosphate-buffered saline. The pellets were resuspended in equal volumes of sample treatment buffer (0.0625 M Tris-HCl, pH 6.8, SDS 2%, glycerol 10%, 2-mercaptoethanol 5%), heated at 100°C in a water-bath for 10 min. SDS-PAGE was performed by the method described by Laemmli,²⁵ with an acrylamide gradient of 10–20% in the running gel. Boiled samples (20 µl) were applied to the gel and electrophoresed in a Protean gel apparatus (BioRad) at a voltage of 60V to run the sample through the stacking gel, after which the voltage was increased to 200 V. Gels were stained with Coomassie Brilliant Blue. The mol. wt standards were phosphorylase B (97.4 kDa), bovine albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa) (BioRad, Richmond, CA, USA). The resulting patterns were scanned with a Bio Image Electrophoresis Analyzer (Millipore), by means of the unweighted pair group method using averages algorithm (UPGMA).¹⁴

Plasmid analysis

Plasmid DNA was isolated essentially as described in Birnboim and Doly's alkaline precipitation method.²⁶ The sample was analysed by horizontal

electrophoresis in agarose 0.7%, and plasmid DNA bands were visualised under UV illumination after being stained with ethidium bromide.

PCR with arbitrary primers

Bacteria were grown on MacConkey agar overnight. Half a colony of each isolate was suspended in 25 µl of distilled sterile water, and boiled for 10 min. After a short centrifugation, 25 µl of a reaction mixture containing 20 mM Tris-HCl (pH 8.8), 100 mM potassium chloride, 3.0 mM magnesium chloride, gelatin 0.1%, 400 µM deoxynucleoside triphosphates and 0.25 µM or 1 µM primer was added, together with 2.5 U of *Taq* polymerase from *Thermus aquaticus* (Boehringer Mannheim, Germany). The reaction was overlaid with oil and subjected to two cycles through the following temperature profile: 94°C for 5 min to denature, 40°C for 5 min for low stringency annealing of primer and 72°C for 5 min for extension. This temperature profile was followed by 40 high stringency cycles: 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. PCR assay was performed in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Emeryville, CA, USA). The sequence of primer used was 5'GTT GTA AAA CGA CGG CCA GT3' (M13 forward primer). Primer was obtained from MedProbe, Oslo, Norway. Amplification products were analysed by electrophoresis in agarose 2% gel and detected by staining with ethidium bromide.

Chromosomal analysis by PFGE

Genomic DNA was prepared in agarose plugs as described previously.²² It was then incubated with a mixture of 0.5 M EDTA, SDS 1% w/v, sarcosyl 1% w/v and 1 mg of pronase E (Serva)/ml for 48 h at 37°C, with a change of solution after 24 h. Protein digestion products were removed by washing the inserts twice for 1 h at 50°C in 10 mM Tris-0.1 mM EDTA (pH 7.5)-1 mM phenylmethylsulphonyl fluoride and three times in Tris-EDTA alone for 1 h each at room temperature. One insert of DNA was incubated overnight with 50 U of *Apa*I or 50 U of *Sma*I (Promega) following the manufacturer's recommendations. The DNA fragments generated were separated in a pulsed-field agarose 1% gel (BioRad) that was prepared and run in Tris-borate-EDTA (TBE) buffer on a contour-clamped homogeneous field apparatus (CHEF-DR2; BioRad). The conditions for electrophoresis were 200 V for 24 h, with pulse time ranging from 5 to 8 s. Thereafter, the gels were stained with ethidium bromide and photographed.

Discriminatory index

The index of discriminatory power was calculated following Hunter and Gaston's equation.²⁷

Reproducibility

This was the ability of a technique to yield the same result when the same strain was tested three times and was classified as moderate, good and excellent.

Results

Biotyping

The API-20NE system identified all isolates as *A. baumannii*, with three different analytical profile index numbers: 0041073, 0041051; 0041473. Changes occurred in the gluconate, adipate and phenylacetate reactions. Seven biotypes were observed among the 92 *A. baumannii* studied by a six carbon-source system. Biotypes 2, 9 and 12 represented 89.1% of *A. baumannii* strains (table I).

Antimicrobial susceptibilities

Table II shows the antibiotic susceptibilities of the strains isolated. Almost all the isolates were resistant to ampicillin, and > 50% were also resistant to piperacillin, cefotaxime, and ceftazidime. Susceptibility to gentamicin, tobramycin, amikacin and netil-

Table II. Antimicrobial susceptibility patterns of *A. baumannii* isolates

Antibiotic	I	II	III	IV	V	VI	VII	VIII
Ampicillin	R	R	R	R	R	R	R	R
Cefotaxime	S	R	MS	R	MS	MS*	MS	R
Cefoxitin	R	R	R	R	R	R	R	R
Ceftriaxone	S	MS	S	MS	MS	MS*	R	R
Cefuroxime	MS	R	R	R	R	MS	R	R
Cefalothin	R	R	R	R	R	R	R	R
Mezlocillin	S	R	R	MS	MS	MS*	R	R
Piperacillin	S	R	R	MS	MS	MS*	R	R
Chloramphenicol	R	R	R	R	R	R	R	R
Gentamicin	S	R	R	R	R	S	R	R
Netilmicin	R	S	R	S	R	S	S	R
Tobramycin	S	R	I	R	R	S	R	R
Amikacin	S	R	S	S	S	S	R	R
Ciprofloxacin	S	S	R	S	S	S	R	R
Imipenem	S	S	S	S	S	S	S	S

R, resistant, I, intermediate, S, susceptible, MS, moderately susceptible.

* Variable susceptibility to these antibiotics.

micin enabled the definition of seven phenotypic resistance patterns; among those, GENs, TOBs, AMKs, NETr (group I), GENr, TOBr, AMKr, NETs (group II), GENs, TOBs, AMKs, NETs (group VI), were the most frequently encountered. All strains were

Table I. Classification of *A. baumannii* isolates by different epidemiological markers

Hospital	Laboratory no.* (number of isolates)	Biotyping		Antibiogram	Plasmid profile	Protein patterns	PFGE type	AP-PCR profile
		API-profile NE	Biotype					
H. Clinico y Provincial	41 (21)†	0041051	12	II	a	B1	7c	7
	36 (17)†	0041073	9	II	f	B1	3	14
	661 (5)	0041051	12	VII	b	B1	7b	7
	724 (5)	0041073	9	VIII	e	A1	6	2
H. Valle de Hebron	74 (1)	0041473	9	VI	g	B1	14	5
	86 (2)	0041473	9	VI	—†	F	9	15
	83 (1)	0041473	6	VI	—	B2	15	8
	58 (2)	0041473	6	VI	—	D	8	6
	61 (1)	0041473	12	VI	—	B1	4	4
	92 (1)	0041473	11	VI	—	B2	20	13
	60 (1)	0041473	8	VI	d	E	12	3
	68 (1)	0041473	9	VI	—	C	13	12
	65 (1)	0041473	2	VI	—	B1	10	8
	72 (1)	0041473	6	VI	—	D	11	1
	88 (3)	0041051	12	II	a	B1	7d	7
	93 (2)	0041051	12	II	a	B1	7c	7
	67 (1)	0041051	12	II	a	B1	7e	7
	70 (1)	0041051	12	II	a	B1	7a	7
	77 (3)	0041073	9	V	—	A1	1a	2
	78 (1)	0041073	9	V	—	A1	2	2
	81 (1)	0041073	9	V	—	A1	1b	2
	91 (1)	0041073	9	V	—	A1	1c	2
H. Sta Cruz y S. Pablo	31 (5)	0041473	2	III	—	A2	16a	8
	175 (2)	0041473	2	III	—	A2	16b	8
	188 (6)	0041073	9	IV	c	B1	5a	11
	182 (1)	0041073	9	IV	c	B1	5b	11
	183 (1)	0041473	6	VI	—	A1	19	16
	198 (2)	0041473	5	VI	—	B1	21	5
	167 (1)	0041473	9	VI	—	E	17	10
	37 (1)	0041473	8	VI	—	D	18	9

PFGE, pulsed field gel electrophoresis; AP-PCR, polymerase chain reaction with arbitrary primers.

*Strain type.

†Isolates from outbreak.

‡Strains without detectable plasmid DNA.

Fig. 1. Plasmid DNA patterns. Lane M1, *Hind*III-digested λ ; M2, DNA mol. wt marker VI (pBR328 DNA-*Bgl*II + pBR328 DNA-*Hinf*I). Lane a, strains 41, 88, 93, 67 and 70; b, strain 661; c, strains 188 and 182; d, strain 60; e, strain 724; f, strain 36; g, strain 74.

Fig. 2. Whole-cell protein SDS-PAGE patterns. Molecular size standards are shown on the right. Lane 1, strain from group F; 2, strain from group E; 3, strain from group D; 4, strain from group C; 5, strain from group B2; 6 and 7, strains from group A1; 8–11, strains from group B1; 12, strain from group A2.

susceptible to imipenem but 18% were resistant to ciprofloxacin.

Plasmid profiles

Plasmid profile analysis revealed seven profiles (table I) (fig. 1): a, b, c, d, e, f, g, "a" was the predominant pattern (30.4%). No plasmids were

detected in 30.4% of the strains, 24% of strains harboured only one plasmid and 45.6% contained two or more plasmids.

SDS-PAGE protein patterns

One-dimensional SDS-PAGE of whole-cell protein extracts produced patterns containing 30–40 discrete

Fig. 3. Dendrogram showing percentage similarity of eight electrophoretic protein patterns, analysed by the unweighted pair group method using averages (UPGMA) algorithm.

bands with molecular sizes varying from 15 to 100 kDa. The region between 21 kDa and 66 kDa proved to be the most discriminatory (fig. 2).

For the purpose of this study the patterns were arbitrarily coded with capital letters; isolates with a similarity > 90% were given the same capital letter followed by a number (table I). Six patterns (A, B, C, D, E and F) were observed with similarity between them ranging from 68 to 88% (fig. 3).

PCR fingerprinting

The 92 bacterial strains were subjected to PCR with M13 forward primer. Two different primer concentrations were assayed (0.25 μ M and 1 μ M). A decrease in the concentration of primers resulted in an overall synthesis of longer amplicons, and smaller fragments tended to disappear from the DNA banding patterns. However, the primer concentration did not modify the distribution of groups.

PCR analysis revealed 16 different banding patterns (table I). PCR patterns generated between two and seven DNA fragments of 0.3–2.2 kb. The small differences in fingerprinting patterns manifested by minor DNA fragments were demonstrated repeatedly. However, a decrease in the intensity of the longer bands was occasionally observed (fig. 4).

Chromosomal analysis by PFGE

Twenty completely different restriction fragment

Fig. 4. Patterns obtained by PCR with the M13 core sequence primer (primer concentration 0.25 μ M). Lane M, DNA mol. wt marker VI (pBR328 DNA-*Bgl*I + pBR328 DNA-*Hinf*I). Lane 1, strain 72; 2, strains 724, 77, 78, 81 and 91; 3, strain 60; 4, strain 61; 5, strains 74 and 198; 6, strain 58; 7, strains 41, 661, 88, 93, 67 and 70; 8, strains 83, 65, 31 and 175; 9, strain 37; 10, strain 167; 11, strains 188 and 182; 12, strain 68; 13, strain 92; 14, strain 36; 15, strain 86; 16, strain 183.

Fig. 5. Chromosomal analysis by PFGE. Digestion of DNA from *A. baumannii* with *ApaI*. Lane M, λ ladder DNA. Each lane shows the result for a different pattern. Lane 1a, strain 77; 2, strain 78; 1b, strain 81; 3, strain 36; 7a, strain 70; 7b, strain 661; 6, strain 724; 7c, strains 41 and 93; 11, strain 72; 9, strain 86; 10, strain 65; 7d, strain 88; 8, strain 58; 7e, strain 67; 1c, strain 91; 16a, strain 31; 16b, strain 175; 5a, strain 188; 17, strain 167; 18, strain 37; 19, strain 183; 5b, strain 182; 12, strain 60; 13, strain 68; 14, strain 74; 15, strain 83; 20, strain 92; 4, strain 61.

Table III. Reproducibility and discriminatory index of six methods used to type 92 isolates of *A. baumannii*.

Typing system	Number of types	Discrimination index	Reproducibility
Biotyping (API-20NE)	3	0.659	+
Biotyping (carbon source system)	7	0.736	+
Antimicrobial susceptibility (AS)	8	0.844	+
Plasmid profile analysis (PPA)	8	0.709	+
Whole-cell protein analysis	8	0.727	++
Pulsed-field gel electrophoresis (PFGE)	29	0.962	+++
Arbitrary primed polymerase chain reaction (AP-PCR)	16	0.872	++
AS and PPA	9	0.854	
AS and PFGE	29	0.962	
AS and AP-PCR	18	0.927	
PFGE and PPA	29	0.962	
PFGE and AP-PCR	29	0.962	
AP-PCR and PPA	18	0.919	

+, moderate; ++, good; +++, excellent.

patterns were observed when the DNA was digested with *ApaI* (table I); each one was designated with an ordinal number. Isolates with similar but not identical patterns, differing in three or fewer restriction fragments, were classified as subtypes of the same strain (e.g. 1a, b, c), while organisms differing by more than three restriction fragments were considered sufficiently divergent to warrant a separate strain designation (fig. 5). The genomic DNA of the strains was also cut by *SmaI*, but the resulting groups were not different

from those obtained with *ApaI*. However, the analysis of genomic DNA of strain no. 198, which was not cut by *ApaI*, generated a pattern different from that of the other isolates when it was cut with *SmaI* (data not shown); therefore, 21 patterns were exhibited.

Discriminatory power and reproducibility

The discriminatory power and the reproducibility of each typing methods are shown in table III. Biotyping,

whole-cell protein and plasmid analysis were the least discriminatory methods (0.659/0.736, 0.727 and 0.709, respectively). Antimicrobial susceptibility and AP-PCR showed moderate discriminatory power (0.844 and 0.872, respectively). Typing based on PFGE of chromosomal DNA appeared to be the most discriminatory method (discrimination index of 0.962).

Discussion

A significant increase in the isolation of *A. baumannii* has been noted in Spanish hospitals during the last few years, particularly in intensive care units and among immunocompromised patients. The absence of a gold standard typing system for *A. baumannii* is a major problem in epidemiological studies of this organism. Six different typing methods were used to study 92 isolates of *A. baumannii*. Of these, 38 were clinical isolates from a previously defined outbreak,³ in which two different strains (strain type nos. 41 and 36) were involved, and 54 were randomly selected clinical isolates of *A. baumannii* from three hospitals, presumably derived from sporadic cases of infection. The primary epidemiological marker is the accurate identification of a bacterial isolate at the species level. Early attempts in biotyping *Acinetobacter* strains relied on gelatinase production, haemolysis, and acid production from glucose.²⁸ Further attempts at biotyping were published by Towner and Chopade²⁹ who used a commercially available 20-test identification system, which allowed the differentiation of seven biotypes of *A. calcoaceticus* var. *anitratus*, with the predominance of biotype 4041473. In the present study, the API-20NE test system made it possible to differentiate only three possible biotypes (0041073, 0041051, 0041473). More recently, a biotyping system based on the utilisation of six carbon sources allowed Bouvet and Grimont⁶ to identify 19 biotypes of *A. baumannii*, with biotypes 1, 2, 6 and 9 being found most frequently. Biotypes 2, 9 and 12 were the most frequent in our study. The correlation between the API-20NE system and the carbon source utilisation tests was 87%, and both methods had a low discriminatory power—0.659 and 0.736 respectively.

Antibiotic resistance patterns have been used to differentiate strains involved in outbreaks.^{5, 11, 30, 31} Although antimicrobial susceptibility testing is important in detecting new resistant phenotypes, the main limitation of this method as an epidemiological tool is its lack of specificity, as strains with identical antibiograms proved to be quite different by other

methods (e.g., strain nos. 74 and 83), and the converse is also true (e.g., strain nos. 41 and 661). Antibiograms may be considered in initial screening to determine strain relatedness, but other epidemiological markers should be used.

Plasmid profiling has been shown to be a valuable epidemiological tool for typing *A. baumannii*.³²⁻³³ However, it is well known that nosocomial micro-organisms may gain or lose plasmids and that repeated sub-culturing and storing may also result in loss of plasmids. In this study, 30.4% of strains did not contain detectable plasmid DNA, a percentage higher than that reported by Gerner-Schmidt,¹⁷ making it necessary to use this typing method in conjunction with other ones.

One-dimensional SDS-PAGE has been described previously as a useful tool for typing *Acinetobacter* spp.^{14, 15} However, we found that the two strains responsible for the outbreak had the same protein pattern, but they were clearly differentiated by other epidemiological markers. Nevertheless, protein patterns were useful for differentiating strains with identical antibiogram pattern and without plasmids.

Graser *et al.*²¹ amplified DNA of *A. baumannii* with the core sequence of the M13 phage as a single primer and found a discrimination power similar to that of PFGE. Their results contrast with ours, because we found PFGE to have a higher discriminatory power (0.962) than AP-PCR (0.872). Results of PFGE and AP-PCR suggested that one of the strains of the outbreak (strain no. 41) had become an endemic strain in the same hospital and had developed resistance to ciprofloxacin. It also appears that strain no. 41 and several strains from hospital Valle de Hebrón (strain nos 93, 67, 70 and 88) were similar, suggesting inter-hospital transmission.

PFGE of chromosomal DNA enables optimal discrimination of isolates with a single test. The use of other typing methods such as PCR or antibiogram together with PFGE did not increase the discrimination index further (table III). However, PFGE has two disadvantages: first, the prolonged time needed to perform the technique and second, it is necessary to make computerised comparisons of patterns when studying large number of strains. Thus, from our experience, it seems that a combination of an easy and rapid technique such as AP-PCR and a more discriminative but more complex technique such as PFGE may be optimal for epidemiological purposes.

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