CHARACTERISATION AND TYPING OF BACTERIA

A comparative study of ribotyping and arbitrarily primed polymerase chain reaction for investigation of hospital outbreaks of *Acinetobacter baumannii* infection

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Summary. Arbitrarily primed polymerase chain reaction (AP-PCR) and ribotyping were compared in an investigation of an outbreak of *Acinetobacter baumannii* infections. Twenty-five clinical isolates shown previously by other criteria to belong to two different groups, and nine randomly selected *A. baumannii* clinical isolates from other hospitals were investigated. Among the strains analysed, nine different *EcoRI* rRNA gene restriction pattern fingerprints were observed. While similarity was detected between strains of the same group, these fingerprints differed clearly between the two *A. baumannii* groups defined in the outbreak. Two of the nine strains selected randomly had the same ribotype as those strains involved in the outbreak, whereas the remaining seven strains each had a different ribotype. When the strains were tested by AP-PCR with 0.25, 0.5 or 1 μM of M13 forward primer, 10 different profiles were obtained. However, 11 profiles were observed if two different primer concentrations (0.25 and 1 μM) were used. It was concluded that ribotyping and AP-PCR exhibited a similar discriminatory power, although AP-PCR had the additional advantages of speed and simplicity.

Introduction

Acinetobacter baumannii is an established cause of nosocomial infection, mainly in intensive care units.^{1–6} Numerous methods have been proposed for typing *A. baumannii* strains. Antibiograms and commercially available biotyping schemes such as the API 20NE system have been used, but published opinions on their reliability vary and they appear to lack sensitivity.⁷ Phage typing, bacteriocin typing and serotyping are available in only a few reference laboratories.^{8–10} Fingerprinting either of whole-cell proteins or cell-envelope proteins by SDS-PAGE has been used successfully,^{11–14} and several studies^{6, 15, 16} have shown plasmid profiling to be useful for typing, despite the fact that c. 28% of strains isolated do not carry plasmids. DNA restriction fragment length polymorphisms (RFLPs) on pulsed-field gels and ribotyping have also been used as epidemiological markers.^{17–20} These methods are highly discriminatory and reproducible, but their use has been limited because of

the lengthy experimental procedures involved. More recently, the polymerase chain reaction (PCR) has been used in conjunction with primers for the core sequence of phage M13²¹ or enterobacterial repetitive intergenic consensus sequences²² to study *A. baumannii* isolates from an outbreak in a hospital. The results seem promising, as all strains involved in the outbreak studied had the same pattern, but further studies are needed to confirm the sensitivity and reproducibility of the method and its value as an epidemiological tool. As discrimination can be optimised by the use of additional primers or modified assay conditions, or both, the purpose of this study was to compare arbitrarily primed PCR (AP-PCR), with two different primers and sets of amplification conditions, with ribotyping for delineating outbreaks of nosocomial infection caused by *A. baumannii*.

Materials and methods

Bacterial strains

In total, 34 isolates were analysed; 25 had been divided previously into two groups (13 strains in the

Fig. 1. Schematic representation of the ribotypes of *A. baumannii*. A, ribotypes obtained from DNA digested with *EcoRI*. Lane A, strain 54; B, 68; C, 74; D, 77 (representative of group I involved in the outbreak) and 183; E, 88 (representative of group II involved in the outbreak) and 201; F, 6; G, 92; H, 198; I, 31. B, ribotypes obtained from DNA digested with *ClaI*. Lane I, strains 6, 31, 77, 183, and 198; II, 88 and 201; III, 54; IV, 68; V, 92. Lane M indicates positions of DNA molecular size markers.

first group and 12 strains in the second group) involved in an outbreak in an intensive care unit,⁶ while the remaining nine strains (strain nos. 6, 31, 54, 68, 74, 92, 183, 198 and 201) were collected from other Spanish hospitals. Identification of *A. baumannii* was based on standard biochemical reactions and the criteria of Bouvet and Grimont.²³

Isolation of chromosomal DNA

The cell pellet from a 10-ml overnight Luria-Bertani broth culture was resuspended in 400 µl of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, mixed with 40 µl of lysozyme 10 mg/ml, and held at -20°C for 30 min. The bacterial cells were then lysed by gentle mixing with 80 µl of lysis buffer (50 mM Tris-HCl, pH 7.5, 400 mM EDTA, SDS 0.5% w/v, proteinase K 1 mg/ml) at 60°C until the lysate cleared (c. 45 min). The DNA was extracted three times with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform:isoamyl alcohol (24:1), and was then precipitated by adding 1 volume of cold ethanol 96% v/v and holding at -20°C for at least 30 min. After washing once with ethanol 70% v/v, the purified DNA was pelleted by centrifugation, dried and resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA before quantifying spectrophotometrically at OD₂₆₀.

DNA restriction endonuclease digestion

Purified DNA (6–8 µg) was digested with 25 U of *EcoRI* or *ClaI* overnight as recommended by the manufacturer (Pharmacia). The resulting DNA fragments were separated by horizontal electrophoresis in agarose 0.6% w/v gels in 89 mM Tris-borate, pH 8.5, 1 mM EDTA run at 35 V for 18 h. After electrophoresis, the gels were stained with ethidium bromide 0.5 mg/L and visualised under UV illumination

(230 nm). *Xenorhabdus* strain 278 (obtained from P. A. D. Grimont, Paris, France) chromosomal DNA digested with *EcoRI* was used on each gel as a molecular size standard.

Southern blotting

DNA in gels was depurinated in 0.25 M HCl for 10 min, denatured in 0.5 M NaOH, 1.5 M NaCl for 30 min with gentle shaking, and neutralised in 1.5 M NaCl, 1 M Tris-HCl, pH 8.0, for 30 min with gentle shaking. The fragments were transferred to a nylon membrane (Hybond-N, Amersham) with a Hybaid vacuum blotter, 10 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) as the transfer solution and a vacuum time of 2 h. The membranes were washed once in 2 × SSC, air-dried and cross-linked under UV light for 3 min.

Ribotyping

Pre-hybridisation, hybridisation with acetylaminofluorene-labelled rRNA from *Escherichia coli* (Eurogentec, Brussels, Belgium) and subsequent detection were as recommended by Eurogentec. Briefly, the nylon membranes were pre-hybridised at 65°C for 1 h in a solution comprising 20 × SSC, 50 × FPG (50 × FPG is Ficoll 400 1% w/v, polyvinylpyrrolidone 1% w/v, glycine 1% w/v) and sheared denatured herring sperm DNA 100 µg/ml. The membranes were hybridised at 65°C overnight in a solution containing 2 × SSC, 1 × FPG, 25 mM KH₂PO₄, pH 8.0, 2 mM EDTA, pH 8.0, SDS 0.5% w/v, freshly denatured sheared herring sperm DNA 100 µg/ml and heat-denatured acetylaminofluorene-labelled rRNA 0.5 µg/ml. After hybridisation, the nylon membranes were washed three times in 2 × SSC, SDS 0.1% w/v and once in 2 × SSC. The hybridisation reactions were

Fig. 2. AP-PCR analysis of *A. baumannii* strains with **A**, 0.25 μ M; **B**, 0.5 μ M; or **C**, 1 μ M of M13 forward primer and temperature profile 1. Lane **a**, strain 54; **b**, 68; **c**, 74; **d**, 77 and 79 (representative of group I involved in the outbreak); **e**, 88 and 93 (representative of group II involved in the outbreak); **f**, 6; **g**, 92; **h**, 201; **i**, 198; **j**, 31. The right-hand lane in each panel (labelled "j", "g" and "g", respectively) corresponds to strain 183. Lane M, DNA molecular size markers.

visualised colorimetrically in an immunoenzymic reaction with nitroblue-tetrazolium, 5-bromo-4-chloro-3-indolyl-phosphate and alkaline phosphatase-labelled anti-acetylaminofluorene antibodies (Eurogentec).

AP-PCR reactions

These were performed as follows: one-half of a colony grown on MacConkey agar was resuspended in 25 µl of sterile distilled water and boiled for 10 min. After a short centrifugation step at 15000 *g*, 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% w/v, 400 µM deoxynucleoside triphosphates and 0.5, 1, or 2 µM of primer was added, together with 2.5 U of *Taq* polymerase (Boehringer Mannheim). The primers used were: 5'-GTT GTA AAA CGA CGG CCA GT-3' (M13 forward amplification primer) and 5'-AAC AGC TAT GAC CAT GAT TA-3' (M13 reverse amplification primer), obtained from MedProbe, Oslo, Norway. Each reaction was overlaid with oil and amplified with one of two different temperature profiles: (i) two cycles of 94°C for 5 min, 40°C for 5 min and 72°C for 5 min, followed by 40 high stringency cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min; (ii) 45 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min. Amplification was performed in a DNA thermal cycler Model 480 (Perkin-Elmer Cetus). Amplified DNA products were resolved by electrophoresis in agarose 2% w/v gels containing ethidium bromide 0.5 mg/L.

Results

Ribotyping

*Cla*I and *Eco*RI were compared for their ability to provide clear and discriminative ribotypes of *A. baumannii*. *Cla*I generated poorly discriminative profiles with a small number of rDNA fragments; however, five different ribotypes (designated I–V) of 5–6 bands were distinguished (fig. 1B), comprising bands between 6.0 and 15 kb in size. In contrast, among the 34 *A. baumannii* strains analysed, nine different *Eco*RI

rRNA gene restriction patterns (designated A–I; fig. 1A) were distinguished. The chromosomal fragments containing rRNA genes ranged in size from 2.5–18 kb, with patterns comprising 5–8 bands. The patterns differed by the presence or absence of at least two bands; exceptions were ribotypes E and I, which appeared to be very similar and differed in only a single *c.* 8.5-kb band. Ribotyping revealed that the outbreak isolates (e.g., strain no. 77—ribotype D—and strain no. 41—ribotype E) and two isolates from other hospitals (strain no. 183—ribotype D—and strain no. 201—ribotype E) shared the same ribotype patterns. If the results obtained with both enzymes were combined, the discriminatory power was not increased.

AP-PCR fingerprinting

The 34 *A. baumannii* isolates were subjected to PCR in conjunction with M13 forward primer, M13 reverse primer, or with both. The results obtained with M13 forward primer alone or both primers together were identical, whereas M13 reverse primer produced poorer discrimination when used alone (data not shown). Identical results were obtained with M13 forward primer and either of the two temperature profiles, but a decrease in the primer concentration resulted in an overall synthesis of longer amplicons; smaller fragments tended to disappear from the DNA banding patterns. Ten different banding patterns (designated a–j; fig. 2) were distinguished, comprising 2–7 DNA fragments of 0.1–2.2 kb in size. PCR profiles of strains involved in the outbreak showed the same pattern for strains of the same group, but were clearly different for strains belonging to different groups. Eight of the nine strains collected from other hospitals had a pattern different from the strains involved in the outbreak. The remaining strain (no. 183) had the same AP-PCR pattern as strain no. 92 with a primer concentration of 1 µM (pattern g) or 0.25 µM (pattern j).

The small differences in fingerprinting patterns caused by minor DNA fragments were demonstrated repeatedly. The reproducibility of the technique was determined by testing three strains five times and the remaining strains four times. However, a decrease in the intensity of the larger bands was observed occasionally. The results were reproducible from one assay to another. The same profiles were obtained with purified DNA and DNA obtained direct from a boiled colony suspension (data not shown).

Correlation between ribotyping and AP-PCR

The ribotyping results showed good agreement with the results obtained by AP-PCR (table). While similarity between strains of the same group was detected by ribotyping and AP-PCR, these techniques clearly differentiated between the *A. baumannii* groups defined previously in the outbreak. Similar results were obtained with the remaining strains of the outbreak not shown in figs. 1 and 2. Ribotypes A, B, C, F, H and I each correlated with a different AP-PCR

Table. Comparison of results obtained by ribotyping and AP-PCR for 34 strains of *A. baumannii*

Ribotype	Number of strains with the AP-PCR profile indicated*									
	a	b	c	d	e	f	g	h	i	j
A	1	0	0	0	0	0	0	0	0	0
B	0	1	0	0	0	0	0	0	0	0
C	0	0	1	0	0	0	0	0	0	0
D	0	0	0	12	0	0	1	0	0	0
E	0	0	0	0	13	0	0	1	0	0
F	0	0	0	0	0	1	0	0	0	0
G	0	0	0	0	0	0	1	0	0	0
H	0	0	0	0	0	0	0	0	1	0
I	0	0	0	0	0	0	0	0	0	1
Total	1	1	1	12	13	1	2	1	1	1

*AP-PCR profiles generated with a primer concentration of 1 µM.

profile. No correlation between the two techniques was observed for two strains (nos. 201 and 183). Strain no. 201 had a different AP-PCR pattern to the other strains studied, but had the same ribotyping pattern as one of the groups involved in the outbreak (ribotype E), whereas strain no. 183 had a similar AP-PCR pattern to strain no. 201 (pattern g) but a different ribotype (D). Other epidemiological tools (analysis of chromosomal DNA by pulsed-field gel electrophoresis, plasmid analysis or antibiograms) also demonstrated clearly that these strains were independent from the other strains analysed (unpublished results).

Discussion

Various genotypic markers have been used for epidemiological typing of *A. baumannii* to investigate relationships between clinical isolates. In addition to the ability to discriminate strains within a species, ease of performance and interpretation of the results and the availability of reagents is of major importance in a clinical laboratory. The objective of this study was to investigate the value of AP-PCR in comparison with ribotyping for an epidemiological investigation. Clinical isolates from an outbreak defined previously,⁶ in which two different groups of strains were involved, and randomly selected clinical isolates of *A. baumannii* from other hospitals were used to assess the discriminative power of AP-PCR. The latter isolates were presumably derived from sporadic cases of infection.

Ribotyping with labelled *E. coli* rRNA used to probe the ubiquitous and polymorphic rRNA loci^{24, 25} has acquired wide acceptance as a powerful epidemiological tool. Ribotyping has several advantages, including patterns which are easy to interpret because of the limited number of hybridised fragments, and ribotypes that are stable and reproducible after continued subculture. The importance of the choice of enzyme for ribotyping has been emphasised elsewhere. In the present study, *EcoRI* provided more fragments and a greater diversity in rDNA patterns than *ClaI*. This contrasted with the data of Grimont *et al.*²⁶ who found that *ClaI* generated 13 different patterns from 20 *A. baumannii* strains. Gerner-Smidt¹⁹ analysed 70 strains in the *A. calcoaceticus*-*A. baumannii* complex after digestion with *EcoRI*, *ClaI* and *Sall*, and observed good discrimination by combining the results obtained with all three enzymes, whereas Dijkshoorn *et al.*¹⁸

showed acceptable strain differentiation by ribotyping with *EcoRI* and *HindIII*, with results similar to those obtained by analysis of cell envelope protein profiles. In the present study, two strains selected randomly from other hospitals had the same ribotype as the strains involved in the outbreak. However, further investigation by pulsed-field electrophoresis, plasmid analysis and antibiograms showed that these strains differed clearly from each other and from the rest of the strains analysed.

Ribotyping is a labour-intensive process and requires several days for completion. The AP-PCR method²⁷ is based on the amplification of random DNA fragments with a single primer of arbitrary nucleotide sequence. Vassart *et al.*²⁸ found that a DNA sequence from the genome of bacteriophage M13 could be used to reveal hypervariable mini-satellite sequences in man and bovines, and primers derived from M13 have been used to study DNA polymorphisms in various animals, plants and microorganisms.²⁹ Graser *et al.*²¹ amplified DNA from *A. baumannii* with the core sequence of the M13 phage as a single primer. The discriminative power was similar to that of pulsed-field gel electrophoresis if a primer concentration of 25 μ M was combined with different amplification conditions to those used in the present study, in which a decrease in band intensity was observed with a primer concentration in excess of 10 μ M, and higher discriminatory power was obtained with primer concentrations of 0.25 or 1 μ M. Recently, Struelens *et al.*²² evaluated PCR-mediated fingerprinting with directed primer sets designed to amplify chromosomal regions lying between repetitive multicopy elements that are dispersed widely in eubacterial genomes, e.g., the 126-bp enterobacterial repetitive intergenic consensus (ERIC) sequence. With this set of primers, agreement was observed between the PCR method and the results of macrorestriction analysis by pulsed-field gel electrophoresis when investigating an outbreak of infections caused by *A. baumannii*. In the present study, ribotypes A, B, C, F, H and I each correlated with a different AP-PCR profile, and it was concluded that AP-PCR was a simple and rapid method with a high discriminatory power which can be used as a complementary technique for the epidemiological study of *A. baumannii* clinical isolates.

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