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

J. VILA, A. MARCOS, T. LLOVET*, P. COLL* and T. JIMENEZ DE ANTA

Servei de Microbiologia, Hospital Clinic, Facultat de Medicina, Universitat de Barcelona, Villarroel 170, 08036 Barcelona and * Departament de Genetica i Microbiologia, Universitat Autonoma de Barcelona, Avda. Sant Antoni M. Claret 167, 08025 Barcelona, Spain

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. Numerous methods have been proposed for typing A. baumannii strains. Anti-biograms 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. Phage typing, bacteriocin typing and serotyping are available in only a few reference laboratories. Fingerprinting either of whole-cell proteins or cell-envelope proteins by SDS-PAGE has been used successfully, and several studies 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. 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 M13 or enterobacterial repetitive intergenic consensus sequences 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...
first group and 12 strains in the second group) involved in an outbreak in an intensive care unit, 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.

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 OD 260.

DNA restriction endonuclease digestion

Purified DNA (6-8 µg) was digested with 25 U of EcoRI or CiaI 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 x SSC (1 x 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 x SSC, air-dried and cross-linked under UV light for 3 min.

Ribotyping

Pre-hybridisation, hybridisation with acetylamino-fluorene-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 x SSC, 0.5 x FPG (50 x 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 x SSC, 1 x 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 acetylamino-fluorene-labelled rRNA 0-5 µg/ml. After hybridisation, the nylon membranes were washed three times in 2 x SSC, SDS 0-1% w/v and once in 2 x SSC. The hybridisation reactions were...
Fig. 2. AP-PCR analysis of A. baumannii strains with A, 0.25 μM; B, 0.5 μM; or C, 1 μM 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.
were resolved by electrophoresis in agarose 2% w/v gels containing ethidium bromide 0.5 mg/L.

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 CCG 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

ClaI and EcoRI were compared for their ability to provide clear and discriminative ribotypes of A. baumannii. ClaI 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 EcoRI 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 profile.
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 and 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, 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 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. who found that ClaI generated 13 different patterns from 20 A. baumannii strains. Gerner-Smidt analysed 70 strains of the A. calcoaceticus–A. baumannii complex after digestion with EcoRI, ClaI and SalI, and observed good discrimination by combining the results obtained with all three enzymes, whereas Dijkshoorn et al. 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 method is based on the amplification of random DNA fragments with a single primer of arbitrary nucleotide sequence. Vassart et al. 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. Graser et al. 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 μM 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 μM, and higher discriminatory power was obtained with primer concentrations of 0.25 or 1 μM. Recently, Struelens et al. evaluated PCR-mediated fingerprinting with directed primer sets designed to amplify chromosomal regions lying between repetitive multi-copy 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.

This work was supported by grant PB88/0206 from DGICYT-Spain to J.V. and grant 93/0693 from FISss-Spain to P.C.

References